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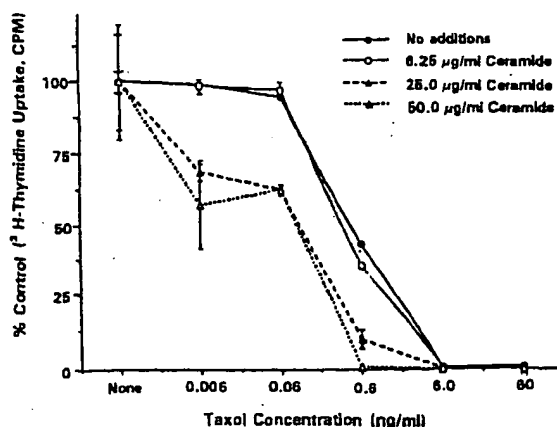
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(54) Title: CERAMIDE AND CHEMOTHERAPEUTIC AGENTS FOR INDUCING CELL DEATH



**(57) Abstract**

This invention provides a method for increasing apoptosis in tumor cells and a method of decreasing a size of a tumor, said methods comprising contacting the tumor cells with: a) an effective amount of at least one antitumor chemotherapeutic agent; and b) an effective amount of a ceramide, sequentially or concomitantly, wherein the apoptosis induced by the combination of the antitumor chemotherapeutic agent and the ceramide is greater than the apoptosis induced by contact of the tumor cells with either the antitumor chemotherapeutic agent alone or the ceramide alone. This invention also provides a method of treating cancer in a subject which comprises a method according to either of the above-described methods. This invention provides a method for treating cancer in a subject comprising administering to the subject an effective amount of at least one antitumor chemotherapeutic agent and an effective amount of at least one ceramide, sequentially or concomitantly. This invention provides a pharmaceutical composition comprising at least one antitumor chemotherapeutic agent in an amount effective to induce apoptosis of tumor cells and at least one ceramide in an amount effective to induce apoptosis of tumor cells and a pharmaceutically acceptable carrier.

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# CERAMIDE AND CHEMOTHERAPEUTIC AGENTS FOR INDUCING CELL DEATH

5 This application claims priority and is a continuation-in-part application of U.S. Serial No. 09/287,884, filed April 7, 1999, the contents of which are hereby incorporated by reference.

10 Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic  
15 citation for these references may be found at the end of this application, preceding the claims.

## BACKGROUND OF THE INVENTION

20 Paclitaxel, an alkaloid compound originally derived from the bark of the Pacific yew tree, is currently used in treatment of breast and ovarian as well as many other types of cancer, including non-small cell lung carcinoma, prostate, head and  
25 neck cancer, and lymphoma. (1-9) The main site of paclitaxel's action is the microtubules of eukaryotic cells, whose functions include formation of the mitotic spindle during cytokinesis, intracellular transport, shape maintenance, cell motility and attachment, and regulation of transmembrane  
30 signals from cell-surface receptors. (10) Paclitaxel acts predominantly by promoting the polymerization of tubulin subunits into microtubules and by preventing depolymerization of the microtubules once they are formed. (11) Paclitaxel has been shown to block cell growth  
35 in the G<sub>2</sub>-M phase of the cell cycle, with subsequent inhibition of mitosis. (12,13) Changes in the structure of microtubules and the mitotic spindle apparatus lead not only to inhibition of cellular division and migration of chromosomes but also to chromosomal breakage. (14)

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Sphingomyelin, a cell membrane component, can be hydrolyzed to ceramide and phosphorylcholine by acid or neutral sphingomyelinase. (15,16) This hydrolysis event initiates an intracellular signalling cascade associated with the stimulation of numerous biological activities, including induction of apoptosis (17-24) and arrest of cell growth in the  $G_0$ - $G_1$  phase. (25-27)

The rationale for combination of paclitaxel and ceramide is based on previous studies that demonstrated activation of Fas expression on leukemic cells by another chemotherapeutic agent, vincristine, which acts primarily by destroying the mitotic spindle in the  $G_2$ -M phase. (28-30) Since ceramide has been reported to mediate, in part, Fas activation, (15,22,23) two agents that appear to converge on some common point in the sphingomyelin/ceramide pathway was evaluated.

Moreover, since these agents act in different phases of the cell cycle, and not all tumor cells can be arrested and/or eliminated in the  $G_2$ -M phase by paclitaxel exposure, additional anti-cancer agents may be needed in the therapeutic regimen. This is supported by the observation that paclitaxel combined with other chemotherapeutic agents in treatment of a variety of cancers, including leukemia, typically produces a stronger tumor cell growth inhibition than a single chemotherapeutic agent. (31-33) Therefore the experiments combine ceramide, a reported  $G_0$ - $G_1$  blocker, with paclitaxel to prevent proliferation of cells that escape the  $G_2$ -M arrest induced by paclitaxel.

This invention provides a method of combination therapy wherein paclitaxel (or other chemotherapeutic agents) and ceramide interact synergistically to induce cytotoxicity and apoptosis in carcinoma cells thereby decreasing the growth of cancer cells.

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# SUMMARY OF THE INVENTION

This invention provides a method for increasing apoptosis in tumor cells comprising contacting the tumor cells with: a)  
5 an effective amount of at least one antitumor chemotherapeutic agent and b) an effective amount of a ceramide, sequentially or concomitantly, wherein the apoptosis induced by the combination of the antitumor chemotherapeutic agent and the ceramide is greater than the  
10 apoptosis induced by contact of the tumor cells with either the antitumor chemotherapeutic agent alone or the ceramide alone, thereby increasing apoptosis in tumor cells.

This invention also provides a method of decreasing a size  
15 of a tumor comprising contacting the tumor with: a) an effective amount of at least one antitumor chemotherapeutic agent and b) an effective amount of a ceramide, sequentially or concomitantly, wherein the induced decrease in size of the tumor is greater than the decrease in size of a tumor after  
20 contacting the tumor with either the antitumor chemotherapeutic agent alone or the ceramide alone, thereby decreasing the size of the tumor.

This invention further provides a method of treating cancer  
25 in a subject which comprises the method according to either of the above-described methods of increasing apoptosis in tumor cells or the above-described of method decreasing a size of a tumor in the subject.

This invention provides a pharmaceutical composition  
30 comprising at least one antitumor chemotherapeutic agent in an amount effective to induce apoptosis of tumor cells and at least one ceramide in an amount effective to induce apoptosis of tumor cells and a pharmaceutically acceptable  
35 carrier.

This invention provides a method for treating cancer in a subject comprising administering to the subject an effective

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amount of at least one antitumor chemotherapeutic agent and an effective amount of at least one ceramide, sequentially or concomitantly.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Effect of paclitaxel (taxol) and ceramide on Jurkat cell growth inhibition. Jurkat cells were maintained as described in Materials and Methods, resuspended at a density of  $1 \times 10^6$  cells/ml, washed by centrifugation for 5 minutes at 1500 rpm, and plated in a final volume of 0.2 ml per well in complete RPMI 1640 medium containing 0% to 10% fetal bovine serum. Paclitaxel (0 to 60 ng/ml and ceramide (0 to 50  $\mu$ g/ml) were added to Jurkat cells either alone or in combination, and proliferation was measured in cpm using a 3-day  $^3$ H thymidine uptake assay. The data are normalized to respective cell control.

15      **Figure 2.**      Effects of Serum on Paclitaxel and Ceramide  
Induced Jurkat Cell Growth Inhibition.    The combined effect  
of the addition of paclitaxel (0.6 ng/ml) and ceramide (25  
μg/ml) on Jurkat cell growth in 0% to 10% fetal bovine serum  
is shown.    Jurkat cells were maintained and treated as  
20    described in Materials and Methods.    Cell growth was assessed  
by a <sup>3</sup>H-thymidine incorporation assay as described in  
Materials and Methods.

Figure 3A-3F. Cell-cycle analysis of Jurkat cells in the presence of paclitaxel and/or ceramide. Cell-cycle analysis was performed at 24 hours because, by 48 and 72 hours, most of the cells (treated with a combination of paclitaxel and ceramide) were hypodiploid, and accurate assessment of the percentage of cells in G<sub>0</sub>-G<sub>1</sub>, S- and G<sub>2</sub>-M phases could not be made. Propidium iodide (0.05 mg/ml) was added to cell cultures in a total volume of 0.5 ml. Samples were analyzed by FACScan (Becton Dickinson, Costa Mesa, CA). Cell-cycle distribution was calculated as a percentage of the total population of both live and hypodiploid cells. (Figure 3A) Untreated Jurkat control cells with no additions of paclitaxel or ceramide; (Figure 3B) with addition of 25 µg/ml of ceramide; (Figure 3C) with 0.6 ng/ml of paclitaxel;

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(Figure 3D) with 0.6 ng/ml of paclitaxel and 25  $\mu$ g/ml of ceramide combined; (Figure 3E) with 6.0 ng/ml of paclitaxel; and (Figure 3F) with 6.0 ng/ml of paclitaxel and 25  $\mu$ g/ml ceramide combined.

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Figures 4A-4F. Wright's stain analysis of Jurkat cells treated with paclitaxel and ceramide. Morphology of paclitaxel- and ceramide-induced apoptosis in Jurkat cells stained with Giemsa/Wright at 48 hours. (Fig. 4A) Untreated Jurkat control cells with no additions of paclitaxel or ceramide; (Fig. 4B) with 25  $\mu$ g/ml of ceramide; (Fig. 4C) with 0.6 ng/ml of paclitaxel; (Fig. 4D) with 6.0 ng/ml of paclitaxel; (Fig. 4E) with 0.6 ng/ml of paclitaxel and 25  $\mu$ g/ml of ceramide combined; and (Fig. 4F) with 6.0 ng/ml of paclitaxel and 25  $\mu$ g/ml of ceramide combined.

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Figure 5A-5H. TUNEL assay for the measurement of paclitaxel- and ceramide-induced Jurkat cell apoptosis. TUNEL assay was performed at both 24 (panel A) and 48 (panel B) hours. Apoptotic cells are demonstrated by the area M1. Fig. 5A and 5E represent untreated control cells; Figs. 5B and 5F represent cells treated with 25  $\mu$ g/ml ceramide; Fig. 5C and 5G represent cells treated with 0.6 ng/ml paclitaxel; and Figs. 5D and 5H represent cells treated with both ceramide and paclitaxel. The percentage of apoptotic cells obtained by flow cytometric analysis is shown below each figure.

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Figures 6A-6B. Effect of Paclitaxel (Taxol) and Ceramide on the growth of Tu138 cells. Tu138 cells ( $2 \times 10^4$ /well) were plated in 96-well culture plates in the presence and absence of paclitaxel (Taxol) or ceramide for a period of 3 days and then subjected to MTT dye assay as indicated in Materials and Methods. The x-axis represents concentrations of paclitaxel (Taxol) (0-6000 ng/ml; Fig. 6A) and ceramide (0-50  $\mu$ g/ml;

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Fig. 6B) and the y-axis represents optical density (540 nm) of live cells that incorporated MTT dye. Each data point represents an average and a standard deviation of triplicate determinations.

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Figure 7. Time Kinetics of the Combined Action of Paclitaxel (Taxol) and Ceramide. Tul38 cells were cultured as indicated in the Figure 6 legend in the presence and absence of paclitaxel (Taxol) (600 ng/ml) and/or ceramide (25  $\mu$ g/ml). The x-axis represents optical density of incorporated dye and y-axis represents the time of measurements at 24, 48 and 72 hours.

10

Figure 8.  $ED_{50}$  Isobologram Analysis of Synergistic Action of Paclitaxel (Taxol) and Ceramide. For the construction of isobologram,  $ED_{50}$  were determined individually from seven independent experiments performed at various dilutions of paclitaxel and ceramide by best fit analysis. Also, a line plot was generated by calculations of  $ED_{50}$  through  $ED_{50}$  values for both paclitaxel and ceramide. The x-axis represents paclitaxel (Taxol) concentrations necessary to generate  $ED_{50}$  in combination with ceramide concentrations plotted on the y-axis. The scatter plot is the result of calculated  $ED_{50}$  values from seven independent experiments representing combined exposures of paclitaxel and ceramide.

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Figure 9: Paclitaxel (Taxol) and Ceramide Induced Cell Cycle Progression and Flow Cytometry Analysis of Tul38 cells. Tul38 cells at a density of  $0.5 \times 10^6$ /ml were cultured in the presence and absence of paclitaxel (Taxol) (600 ng/ml) and/or ceramide (25  $\mu$ g/ml) for 24 (panel A) and 48 (panel B) hours in either 6-well culture plates to T-25 flasks. At the end of the incubation period, cells were trypsinized, washed and subjected to a flow cytometric analysis as described in Materials and Methods. The x-axis of the scans represents DNA content and y-axis represents the number of cells. The analysis of the acquired samples with the use of "Modfit"

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software is shown underneath each treatment as the percent population of viable cells in various phases of the cell cycle.

5     **Figure 10.** TUNEL Assay for the Measurements of Paclitaxel (Taxol) and Ceramide Induced Apoptosis of Tu138 cells. For the measurement of apoptosis, Tu138 cells were cultured with or without paclitaxel (Taxol) (600 ng/ml) and/or ceramide (25  $\mu$ g/ml) as described Figure 9. The analyses of acquired  
10     samples were based on an antibody binding to DNA fragments shown as fluorescence on x-axis and cell count on y-axis. For the positive control, Tu138 cells were treated with DNase for 10 minutes at room temperature prior to the acquisition on FACScan. The percent shown underneath each scan was  
15     obtained by the use of CELL Quest software (Becton Dickinson, CA) and is represented by M1.

20     **Figure 11.** Growth of Human Squamous Carcinoma Cells in Nude Mice. Tumor growth of head and neck squamous carcinoma (TU138) was significantly reduced by treatment with a combination of paclitaxel and ceramide.

25     **Figure 12.** Excised Tumor Weights in Nude Mice. Tumor growth of head and neck squamous carcinoma (TU138) was significantly reduced by treatment with a combination of paclitaxel and ceramide compared with tumor growth reduction in control, nude mice treated with paclitaxel alone, and nude mice treated with ceramide alone.

30     **Figure 13.** Growth of Human Squamous Carcinoma Cells in Nude Mice. A significant reduction (88%) in tumor weight was achieved with subcutaneous administration of paclitaxel and ceramide for a period of 5 weeks near the site of tumor (Tu138) implantation in nude mice in comparison to control  
35     only treated mice.

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Figure 14. Effect of Paclitaxel and Ceramide on Tu-138 Cell-Tumor Weight. A 79% decline in tumor size of paclitaxel and ceramide treated Tu138 tumors in nude mice was observed on day 45; ceramide-only treated mice demonstrated a slight  
5 decrease in tumor size at week 5; Tu-138 cell tumors grew almost equally in control and paclitaxel treated mice.

Figure 15. Tu-138 Tumor Cell Growth in Nude Mice: Intraperitoneal Treatment with Paclitaxel and Ceramide.  
10 Intraperitoneal delivery of paclitaxel and ceramide resulted in 60% tumor size reduction within 4 weeks of administration compared to control (vehicle alone); paclitaxel alone reduced tumor size by 29%, and ceramide alone had no significant effect.

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#### DETAILED DESCRIPTION OF THE INVENTION

This invention provides a method for increasing apoptosis in tumor cells comprising contacting the tumor cells with: a)  
5 an effective amount of at least one antitumor chemotherapeutic agent and b) an effective amount of a ceramide, sequentially or concomitantly, wherein the apoptosis induced by the combination of the antitumor chemotherapeutic agent and the ceramide is greater than the  
10 apoptosis induced by contact of the tumor cells with either the antitumor chemotherapeutic agent alone or the ceramide alone, thereby increasing apoptosis in tumor cells.

As used herein "increasing apoptosis" is defined as an  
15 increase in the rate of programmed cell death, i.e. more cells are induced into the death process as compared to exposure (contact with) either the chemotherapeutic agent alone or the ceramide alone. Increasing apoptosis also includes the inhibition of cell division which results in a  
20 decrease in the total number of viable tumor cells.

As used herein "contacting tumor cells" is defined as exposing the tumor cells to combination therapy, i.e. administering to the tumor cells directly or indirectly,  
25 chemotherapeutic agent(s) and ceramide by local, regional or syatemic means.

As used herein a "ceramide" is any N-acylsphingosine. Ceramides include sphingolipids in which the sphingosine is acylated with a fatty acid acyl CoA derivative to form an N-acylsphingosine. Ceramide may be either naturally occurring or chemically synthesized. Preferably, the carbon chain length is less than 18 carbons. Examples include C6-ceramide (N-hexanoyl-D-sphingosine), C2-ceramide  
30 (N-acetyl-D-sphingosine), C8-ceramide (N-octyl-D-sphingosine) and C16-ceramide (N-palmitoyl-D-sphingosine. Other ceramides are known to one of skill in the art.

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Preferably, the ceramide (which is lipid soluble) is water soluble or made water soluble to enable contact with the tumor cells in a subject. Ceramide (6%) may be solubilized initially in alcohol and then subsequently diluted in saline or a cremophore.

This invention also provides a method of decreasing a size of a tumor comprising contacting the tumor with: a) an effective amount of at least one antitumor chemotherapeutic agent and b) an effective amount of a ceramide, sequentially or concomitantly, wherein the induced decrease in size of the tumor is greater than the decrease in size of a tumor after contacting the tumor with either the antitumor chemotherapeutic agent alone or the ceramide alone, thereby decreasing the size of the tumor.

As used herein "decreasing the size of a tumor" is defined as a reduction in the size of a tumor; the reduction is accomplished by reducing the number of proliferating tumor cells in the tumor, i.e. reducing cell division of the tumor cells, and by inducing cytotoxicity or cell death (apoptosis) of existing tumor cells. Accordingly, tumor growth is arrested or prevented.

In an embodiment of either the above-described method of increasing apoptosis in tumor cells or the above-described method of decreasing a size of a tumor via combination therapy with a chemotherapeutic agent (e.g. paclitaxel) and ceramide, the tumor cells may be or the tumor is composed of cancer cells selected from the group consisting of, but not limited to, leukemic cells, prostate cancer cells, pancreatic cancer cells and squamous cell carcinoma cells, breast carcinoma cells, melanoma cells, basal cell carcinoma cells, neuroblastoma cells, glioblastoma multiforme cells, myeloid leukemic cells, colon carcinoma cells, endometrial carcinoma cells, lung carcinoma cells, ovarian carcinoma cells, cervical carcinoma cells, osteosarcoma cells and lymphoma cells. In further embodiments of the above-described methods,

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the ceramide may be a C2-ceramide, C6-ceramide, C8-ceramide, C16-ceramide, or a higher order of ceramides.

5 In another embodiment of either the above-described method of increasing apoptosis in tumor cells or the above-described of method decreasing a size of a tumor, the tumor cells are or the tumor is contacted first with at least one antitumor chemotherapeutic agent (e.g. paclitaxel) and subsequently contacted with the ceramide.

10 This invention further provides a method of treating cancer in a subject which comprises the method according to either of the above-described methods of increasing apoptosis in tumor cells or the above-described of method decreasing a size of a tumor in the subject, wherein the tumor cells or the tumor are present in the subject.

15 In an embodiment of the method of treating cancer in a subject according to any of the above-described methods, wherein the ceramide may be a C2-ceramide, C6-ceramide, C8-ceramide, C16-ceramide or a higher order of ceramide. In further embodiments more than one ceramide may be administered to the subject.

25 In another embodiment of either the above-described method of increasing apoptosis in tumor cells or the above-described of method decreasing a size of a tumor via combination therapy with a chemotherapeutic agent (e.g. paclitaxel) and ceramide, the antitumor chemotherapeutic agent is paclitaxel or compounds structurally related to the paclitaxel family of compounds, e.g. alkaloids. The antitumor chemotherapeutic agent in any of the above-described methods may also include but not be limited to chemotherapeutic agents such as doxorubicin, cis-platin, cyclophosphamide, etoposide, 30 vinorelbine, vinblastin, tamoxifen, colchicin, 2-methoxyestradiol. In further embodiments of the above-described methods the paclitaxel may be used together with another antitumor chemotherapeutic; combinations of any of

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the above-listed antitumor chemotherapeutic agents may be used.

5 In still further embodiments of either the above-described method of increasing apoptosis in tumor cells or the above-described of method decreasing a size of a tumor via combination therapy with a chemotherapeutic agent (e.g. paclitaxel) and ceramide, the contacting with the antitumor chemotherapeutic agent (exposure of the tumor cells to the  
10 antitumor chemotherapeutic agent) is effected by cremophore delivery or by liposome-mediated delivery and the contacting with (exposure to) the ceramide is effected by alcohol-mediated delivery or liposome-mediated delivery.

15 As used herein a "cremophore" is a solvent that permits solubilization of a drug or compound. Various cremophores are well known to one of skill in the art, including but not limited to oil based solvents.

20 In still further embodiments of any of the above-described methods of combination therapy, the route of administration (contacting the tumor cells) effected by cremophore-mediated delivery, alcohol-mediated delivery or liposome-mediated delivery of the ceramide(s) or of the antitumor  
25 chemotherapeutic agent(s) may be selected from but not limited to any of the following routes of administration: intravenous, intraperitoneal, intra-theal, intralymphatical, intramuscular, intralesional, parenteral, epidural, or subcutaneous administration; by infusion, by aerosol  
30 delivery; or by topical, oral, nasal, anal, ocular or otic delivery.

This invention also provides a pharmaceutical composition comprising at least one antitumor chemotherapeutic agent in  
35 an amount effective to induce apoptosis of tumor cells and at least one ceramide in an amount effective to induce apoptosis of tumor cells and a pharmaceutically acceptable carrier.

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5 This invention provides a method for treating cancer in a subject comprising administering to the subject an effective amount of at least one antitumor chemotherapeutic agent and an effective amount of at least one ceramide, sequentially or concomitantly.

10 As used herein an "effective amount" is defined as an amount of antitumor chemotherapeutic agent and the ceramide which provides the maximum apoptosis of tumor cells at the least toxicity to nontumor cells. The effective amount may be measured as the concentration of antitumor chemotherapeutic agent and ceramide which induces a 50% death rate (ED 50) of tumor cells.

15 In an embodiment of the above-described method for treating cancer in a subject, at least one antitumor chemotherapeutic agent and subsequently at least one ceramide is administered to the subject.

20 In another embodiment of the above-described method for treating cancer in a subject, at least one ceramide and subsequently at least one antitumor chemotherapeutic agent is administered to the subject.

25 In a further embodiment of the above-described method for treating cancer in a subject, the antitumor chemotherapeutic agent is paclitaxel and the ceramide is C6-ceramide. In other embodiments the antitumor chemotherapeutic agent is a  
30 compounds structurally related to the paclitaxel family of compounds, e.g. alkaloids. The antitumor chemotherapeutic agent in any of the above-described methods may also include but not be limited to chemotherapeutic agents such as  
35 doxorubicin, cis-platin, cyclophosphamide, etoposide, vinorelbine, vinblastin, tamoxifen, colchicin, 2-methoxyestradiol. In further embodiments of the above-described methods the paclitaxel may be used together with



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another antitumor chemotherapeutic; combinations of any of the above-listed antitumor chemotherapeutic agents may be used. In still further embodiments of the above-described methods the ceramide may be a C2-ceramide, C8-ceramide or a  
5 higher order of ceramide. In additional embodiments more than one ceramide may be administered to the subject.

This invention will be better understood from the Experimental Details which follow. However, one skilled in  
10 the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

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## EXPERIMENTAL DETAILS

### METHODS AND MATERIALS

#### 5 1st Series of Experiments

Paclitaxel-induced Apoptosis in Jurkat, a Leukemic T Cell Line, Is Enhanced by Ceramide

10 It is hypothesized that the lipid second messenger, ceramide, and microtubule-directed chemotherapeutic agents might engage converging pathways in inducing apoptosis. The studies described herein demonstrate that simultaneous treatment of Jurkat cells with paclitaxel and ceramide enhanced  
15 paclitaxel-induced cell growth inhibition. Cell-cycle analysis indicated a significant increase in the hypodiploid population over that observed with paclitaxel treatment alone. Morphologic evaluation and a TUNEL assay confirmed a  
20 dramatic increase in apoptosis in Jurkat cells treated with the combination of these two agents. This is the first demonstration that paclitaxel and ceramide interact in a supra-additive manner to decrease leukemic T-cell growth, suggesting a possible application of paclitaxel and ceramide in combination therapy.

25

### MATERIALS AND METHODS

Cell Proliferation Assays. Jurkat, a human acute T cell leukemia line (American Type Culture Collection, Rockville,  
30 MD) was routinely maintained in an incubator at 37°C and 5% CO<sub>2</sub> at a density of 0.5 x 10<sup>6</sup> cells/ml in complete RPMI 1640 medium containing 1% L-glutamine, 1% penicillin-streptomycin (GIBCO Biological Research Laboratories, Grand Island, NY) and 10% - 20% fetal bovine serum (Atlanta Biologicals,  
35 Atlanta, GA). For proliferation assays, cells were washed twice and resuspended in complete RPMI 1640 medium at a density of 0.5 x 10<sup>6</sup> cells/ml in a final volume of 0.2 ml per well in a 96-well microtiter plate. Paclitaxel (Bristol-Myers Squibb Co., Princeton, NJ) and ceramide were added as

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indicated below. DNA synthesis was assessed by the addition of  $^3\text{H}$ -thymidine (1  $\mu\text{Ci}/\text{well}$ ; specific activity - 6.7 Ci/mmol; ICN Pharmaceuticals, Costa Mesa, CA) uptake 24 hours before the end of the 72 hour growth period at a final concentration of 1  $\mu\text{Ci}/\text{well}$ . Cells were harvested on glass microfibre filters (Whatman Co., Maidstone, England) with a cell harvester (Cambridge Technology, Cambridge, MA) and counted on a LKB Wallac 1214 RackBeta 'Excel' Beta spectrophotometer (Wallac Oy, Turku, Finland) after the addition of an 0.5-ml EcoLite (ICN Pharmaceuticals, Costa Mesa, CA) scintillation cocktail.

Paclitaxel and ceramide treatment of cell cultures. Paclitaxel was added to Jurkat cell cultures at final concentrations ranging from 0.006 to 6000 ng/ml. Exogenous ceramide ( $\text{C}_6\text{-N-hexanoyl-D-sphingosine}$ ; Sigma Chemicals, St. Louis, Mo) was dissolved in 100% ethanol, and, after dilution with culture medium, was added to cell cultures at final concentrations ranging from 5 to 25  $\mu\text{g}/\text{ml}$ . An equal proportion of 100% ethanol was diluted in complete RPMI 1640 medium and added to cell cultures in the absence of ceramide to eliminate the possibility of alcohol cytotoxicity. To examine the effects of combination treatment, paclitaxel (0.6 ng/ml and 6.0 ng/ml) and ceramide (25  $\mu\text{g}/\text{ml}$ ) were added simultaneously at the beginning of cell culture.

Cell Cycle Analysis. Cells were cultured in 6-well plates (Falcon Products, St. Louis, Mo) at a density of  $0.5 \times 10^6$  cells/ml and incubated in parallel experiments with paclitaxel alone at 0.6 ng/ml and 6.0 ng/ml, ceramide alone at 25  $\mu\text{g}/\text{ml}$ , and paclitaxel and ceramide in combination at 0.6 ng/ml and 6.0 ng/ml and 25 ( $\mu\text{g}/\text{ml}$  respectively. A 3 ml aliquot was obtained from each 6-well plate at the indicated times. Cells were centrifuged twice at 1500 rpm for 5 minutes and resuspended in saline, labeled with 0.5 ml of propidium iodide (0.05 mg/ml), and analyzed by FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA) using ModFit LT software for cell-cycle analysis essentially

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as described by Look et al. (34) Cell growth was calculated as a percentage of the total population of both live and hypodiploid cells. Pie graphs (Figures 3A-F) were generated and normalized to a control value of 100%.

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Histologic Evaluation. Cytospins (Shandon Incorporated, Pittsburgh, PA) of Jurkat cells treated with 0.6 ng/ml and 6.0 ng/ml paclitaxel and/or 25  $\mu$ g/ml of ceramide were generated by centrifuging the cells onto glass slides (200-ml aliquot/slide) for 3 minutes at 750 rpm. Wright's stains of all slides were performed using an automated Hemastainer (Geometric Data, Wayne, PA). Slides were analyzed microscopically, photographed (Olympus BH 2, Tokyo, Japan) at 100, 200, and 400 power, and evaluated for morphological changes.

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Terminal deoxynucleotide transferase (TdT) mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) assay. Jurkat cells were cultured in 6-well plates at a density of  $0.5 \times 10^6$ /ml in a total volume of 5 ml, in RPMI complete medium containing 10% fetal bovine serum at 37°C; the cells were treated with and without 0.6 ng/ml of paclitaxel, 12.5 and 25  $\mu$ g/ml of ceramide, or a combination of the two. At 24 and 48 hours, DNA strand breaks were labeled with TUNEL assay (In Situ Cell Death Detection Kit, Fluorescein; Boehringer Mannheim, Indianapolis, IN), and cellular apoptosis was measured by flow cytometry.

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## 30 RESULTS

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Initial experiments focused on the effects of varying concentrations of ceramide on paclitaxel-mediated inhibition of Jurkat cell growth, as measured by  $^3$ H-thymidine uptake assay. Jurkat cells were plated in the presence of different amounts (0 to 50  $\mu$ g/ml) of  $C_6$ -ceramide and/or paclitaxel (0 to 60 ng/ml) in RPMI-1640 culture medium containing 10% fetal bovine serum. As indicated in Fig. 1, paclitaxel at

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concentrations higher than 0.06 ng/ml effectively blocked thymidine incorporation of Jurkat cells. The calculated  $ED_{50}$  for paclitaxel alone (n=5) was approximately 0.5 ng/ml in a 3-day exposure assay. Ceramide addition produced a marked enhancement of paclitaxel-mediated growth inhibition at 0.6  
5 ng/ml, increasing from 56.5% to 90.1% and >99% (with 25 and 50  $\mu$ g/ml of ceramide, respectively). Ceramide added alone did not cause significant Jurkat cell growth inhibition ( $\leq 6\%$ ) at all test concentrations (Fig. 1, Table 1).

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TABLE 1

Taxol and ceramide-induced growth inhibition<sup>a</sup>

Additions	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
None	382839 ± 23416	444400 ± 17569	258826 ± 39243	301150 ± 25159	242529 ± 38947
Ceramide 25 µg/ml	378245 ± 28689	415740 ± 6168	301993 ± 67599	259230 ± 54914	189052 ± 3534
Taxol 0.6 ng/ml	52675 ± 7478	95563 ± 6427	22771 ± 5113	89621 ± 17579	98694 ± 32045
Taxol 0.6 ng/ml and Ceramide 25 µg/ml	24149 ± 2688	48356 ± 2777	1652 ± 21	70929 ± 16035	449 ± 143
Taxol 6.0 ng/ml	16592 ± 782	33443 ± 17976	13400 ± 849	35466 ± 7576	10295 ± 353
Taxol 6.0 ng/ml and Ceramide 25 µg/ml	11509 ± 965	8536 ± 608	1262 ± 288	8907 ± 2484	249 ± 147

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<sup>a</sup>This table demonstrates that ceramide consistently enhances taxol mediated cell growth inhibition over a series of several experiments. The numbers displayed are actual [<sup>3</sup>H]thymidine uptake counts ± SD (cpm), obtained from triplicate determination. A paired student t test demonstrated statistical significance within a 95% confidence interval.

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Since earlier studies indicated that variation of serum concentration affects ceramide-mediated growth inhibition, (26,35) Jurkat cell growth inhibition in cells cultured in the presence of ceramide and/or paclitaxel with varying amounts of serum was investigated (Fig. 2). Ceramide-mediated growth inhibition was more pronounced (>99%) at low serum (1%) levels compared with that observed in high serum ( $\geq 5\%$ ) conditions. Growth inhibition in cultures treated with paclitaxel alone was unaffected by variation of serum levels from 10% to 1%. Combination of paclitaxel (0.6 ng/ml) and ceramide in 10% serum inhibited growth  $\geq 61\%$  more than paclitaxel alone (n=5) (Fig. 2).

In the next series of experiments it was sought to establish the effect of ceramide on paclitaxel-induced arrest of cell cycle growth measured at 24 hours (Figures 3A- 3F). Analysis of untreated Jurkat cells at the beginning of culture revealed that 51.7% of the cell population was in  $G_0$ - $G_1$  phase, 32.9% in S phase, and 9.4% in  $G_2$ -M phase (data not shown). After 24 hours, the percentage of cells in  $G_0$ - $G_1$  decreased by 18.4% with a concurrent equivalent S-phase augmentation, which suggested significant cell growth after the addition of fresh culture medium (Fig. 3A). The cell cycle pattern following ceramide treatment in the presence of 10% fetal bovine serum did not differ from the non-ceramide-exposed control at 24 hours (Fig. 3B).

The addition of 0.6 or 6.0 ng/ml paclitaxel alone at 24 hours resulted in the accumulation of 22.2% and 38.2%, respectively, of the total cell population in the  $G_2$ -M phase compared with 12.6% of the untreated cell control. This 2-3 fold increase in the  $G_2$ -M cell population was followed by 13.3% or 23.5% decrease of cells in the  $G_0$ - $G_1$  phase, thus confirming the known action of paclitaxel (Figs. 3C and 3E).

In Jurkat cells treated with a combination of either 0.6 or 6.0 ng/ml paclitaxel and ceramide, cell populations decreased to approximately 10% in  $G_2$ -M phase and 8.8% and 0.9% in S

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phase (in contrast to 31.8% and 27% with paclitaxel alone). Therefore combined treatment seems to eliminate cells from the S- and G<sub>2</sub>-M phases (Figs. 3D and 3F).

5 Flow-cytometric analysis also indicated the presence of hypodiploid (sub-G<sub>1</sub>) cell population (Figs. 3A-3F). Compared with untreated control values of 10%, treatment with 0.6 and 6.0 ng/ml paclitaxel each resulted in hypodiploid populations of approximately 25%. The addition of ceramide to  
10 paclitaxel-treated cultures demonstrated a substantial accumulation (to 60%-79%) of hypodiploid cells. These findings indicate that low thymidine uptake in combined paclitaxel and ceramide-treated cultures is not only the consequence of cell growth arrest but also possibly of cell  
15 death.

To observe the morphological changes induced by paclitaxel, ceramide, and their combination, a Wright's stain analysis of sample cultures was performed after 48 hours' treatment  
20 (Fig. 4). Fig. 4A illustrates the morphological features of normal Jurkat cells at 48 hours. A high nuclear-to-cytoplasmic ratio was evident, and all cell membranes were intact with abundant cytoplasmic granules present. Ceramide-treated cell populations were  
25 morphologically indistinguishable from control cells in 10% serum (Fig. 4B). In contrast, apoptotic bodies were noted amid cell shrinkage and nuclear and membrane blebbing following treatment with 0.6 and 6.0 ng/ml of paclitaxel alone (Figs. 4C and 4D). When ceramide and 0.6 and 6.0 ng/ml  
30 of paclitaxel were added in combination, more extensive features of cell death, including a loss of cell membrane and cytoplasmic contents, were apparent (Figs. 4E and 4F).

The presence of apoptosis in Jurkat cells treated with 0.6  
35 ng/ml of paclitaxel, 12.5 or 25 µg/ml of ceramide, and combinations of both agents was confirmed by TUNEL assay at 24 and 48 hours. Apoptosis in Jurkat cells treated with



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either 0.6 ng/ml of paclitaxel or 12.5 and 25  $\mu$ g/ml of ceramide alone was almost undetectable ( $\leq 3\%$ ) at 24 hours. At 48 hours, exposure of Jurkat cells to 0.6 ng/ml of paclitaxel resulted in 19.6% apoptosis, but no significant apoptosis (0.41% and 0.33%) was observed in samples treated with 12.5 and 25  $\mu$ g/ml of ceramide, respectively, at 48 hours. The combination of 0.6 ng/ml of paclitaxel and 25  $\mu$ g/ml of ceramide resulted in 25.5% and 66.1% TUNEL-positive cells at 24 and 48 hours, respectively, demonstrating supra-additive enhancement of cell death (Figs. 5A-5H).

### DISCUSSION

Although synergy between paclitaxel and many other chemotherapeutic agents, such as vincristine and etoposide,<sup>31-33</sup> has proven effective in cancer cell growth inhibition, this study is the first to characterize ceramide enhancement of paclitaxel-mediated growth inhibition. Paclitaxel-induced cell growth inhibition of Jurkat leukemic cells occurred optimally at an ED<sub>50</sub> of approximately 0.5 ng/ml, in agreement with widely cited findings that identify paclitaxel as a growth-inhibitory agent for many human leukemic cell lines.<sup>36-39</sup> Furthermore, it was observed that the level of growth inhibition by ceramide depends on serum concentration. The observation of the absence of ceramide-induced cell growth inhibition in 10% serum in Jurkat T cells paralleled studies that showed a reduction in ceramide-induced cell growth inhibition in cell lines CMK-7, HL-60, and U937.<sup>35</sup> Interestingly, the enhancement of paclitaxel action by ceramide was not affected by high serum concentrations.

Paclitaxel and ceramide have been reported to arrest the growth of cells in the G<sub>2</sub>-M and G<sub>0</sub>-G<sub>1</sub> phases of the cell cycle, respectively. (12, 13, 25-27) In the cell cycle studies described herein, the percentage of cells in G<sub>0</sub>-G<sub>1</sub> was almost identical in both ceramide-treated and untreated cell cultures, consistent with the lack of effect on <sup>3</sup>H-thymidine

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uptake under these conditions. A significant change was found in the percentage of cells in S phase after paclitaxel treatment, indicating the loss of active DNA synthesis by Jurkat cells. The hypodiploid population of cells treated with the combination of paclitaxel and ceramide increased significantly compared to those treated with paclitaxel or ceramide alone. Paclitaxel addition at 0.6 and 6.0 ng/ml yielded  $G_2$ -M/ $G_0$ - $G_1$  ratios of 1.11 and 3.9, respectively, consistent with the known action of paclitaxel as a  $G_2$ -M blocker of the cell cycle. (12,13) Combinations of paclitaxel (0.6 and 6.0 ng/ml) and ceramide (25  $\mu$ g/ml) demonstrated  $G_2$ -M/ $G_0$ - $G_1$  ratios of 0.54 and 1.0, respectively, indicating that the cell population being eliminated was also from the  $G_2$ -M phase, in addition to S phase, resulting in cell death. These results were consistent with TUNEL assay and also by histological evaluation utilizing the Giemsa-Wright (40) staining technique.

Previous studies have demonstrated activation of Fas expression on leukemic cells by another mitotic spindle agent, vincristine, which differs from paclitaxel in that it acts by degrading the mitotic spindle in  $G_2$ -M phase. (28-30) Ceramide enhancement of paclitaxel-mediated apoptosis may possibly be attributed to a mechanism similar to that of the activation of Fas on the surface of Jurkat cells. (15,22,41-42) It is hypothesized that paclitaxel enhances intracellular ceramide production by increasing acid sphingomyelinase activity by the engagement of either Fas receptor or another homologous TNF receptor such as DR4. (43) This enhancement of intracellular ceramide levels is further augmented by the addition of exogenous ceramide. Recent studies have shown that paclitaxel is capable of upregulating Fas ligand expression. (29,44) It may be possible that paclitaxel/ceramide mediated enhancement is due to upregulation and release of Fas ligand from the surface of Jurkat cells and into the surrounding cell culture media, which can then induce cellular suicide by ligating Fas on the cell membrane. Such a mechanism has been proposed in

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activation-induced death of Jurkat cells whereby APO2/TRAIL ligand, a TNF homologue, binds to newly discovered DR4 receptor. (43)

5 While the role of Fas mediated events during paclitaxel exposure is unknown, recent reports have disputed the role of the generation of ceramide in apoptosis by suggesting that ceramide is either not involved in Fas-mediated apoptosis or is not a critical component of the initial apoptotic events and may instead act downstream of the cascade. (45-47)

10 However, none of these studies measured intracellular ceramide levels at high serum concentrations, which, the present study shows, may profoundly affect the role in leukemic cell apoptosis. The present study suggests that cell apoptosis mediated by paclitaxel and ceramide may be linked by a common signal transducing pathway leading to cell death.

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## 2nd Series of Experiments

Effects of Paclitaxel and Ceramide on Human Squamous Carcinoma Cell Line Tul38 in Vitro and in Vivo

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The purpose of this study was to observe in vitro and in vivo effects (additive or synergistic) of combining paclitaxel and ceramide in inducing cytotoxicity and apoptosis in human squamous carcinoma cells (Tul38). Synergistic cytotoxic and apoptotic action of these two agents in vitro in other neoplastic cell lines have been previously demonstrated.

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## METHODS

The effects of paclitaxel and ceramide on cell growth of Tul38 was evaluated by MTT dye assay (see infra p. 35), cell cycle progression by flow cytometry (see infra pp. 35-36), cell apoptosis by TUNEL assay (see infra pp. 35-36) and tumor growth and weight in nude mice (see infra pp. 37-38). To corroborate in vitro findings Tul38 cells were planted subcutaneously on nude mice. Treatment with paclitaxel (120  $\mu$ g/0.1mL) + ceramide (500  $\mu$ g/0.2mL) was begun on day 4 with thrice weekly injections administered subcutaneously near the tumor site for 5 weeks. Other treatment groups included: vehicle only, paclitaxel alone and ceramide alone. Tumor size was measured once a week. All groups of mice were sacrificed on day 45 and excised tumors were weighed.

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## RESULTS

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Cell growth studies showed synergistic growth inhibition with paclitaxel + ceramide and was confirmed by a 50% isobologram analysis in six separate experiments (p value <0.05). The TUNEL assay also confirmed enhanced apoptosis by the combined treatments. Cell cycle progression studies suggested that the target population of cells was either in the S or G<sub>2</sub>-M phase of the cell cycle. Preliminary in vivo studies showed equivalent growth of Tul38 cell-tumor in control and

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paclitaxel treated mice whereas the ceramide injected group showed a slight decrease in tumor size at week 5. The paclitaxel + ceramide group showed a decline of 70% in tumor size compared to control group. There was also significant reduction in tumor weight (88% in paclitaxel + ceramide group vs. control). The ceramide only group also had a substantial reduction in tumor weight. The excised tumors are being studied to determine tumor cell morphology and cellular apoptosis.

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## DISCUSSION

Paclitaxel and ceramide are synergistic in inducing apoptosis and cytotoxicity in the Tul38 squamous cell line in vitro. In vivo effects on tumor growth and weight are similar suggesting preclinical therapeutic potential of these agents.

## 3rd Series of Experiments

20 COMBINED CYTOTOXIC ACTION OF PACLITAXEL (TAXOL) AND CERAMIDE ON HUMAN TUL38 SQUAMOUS CARCINOMA CELL LINE OF THE HEAD AND NECK

Increased intracellular ceramide levels can be induced by a variety of agents including TNF $\alpha$ , Fas, ionizing radiation and chemotherapeutic agents causing apoptosis in several cell systems. Exogenous ceramide can also induce apoptosis by raising intracellular levels. Paclitaxel (taxol), a highly efficacious chemotherapeutic drug, used in the treatment of recalcitrant ovarian and breast as well as other neoplasms, is now undergoing Phase II trials in squamous cell carcinoma of the head and neck. This study shows that paclitaxel and ceramide both induce apoptosis in a human squamous carcinoma cell line Tul38, with an ED<sub>50</sub> of 1920 $\pm$ 1200 ng/ml and 22 $\pm$ 5  $\mu$ g/ml respectively. Ceramide combined with paclitaxel induced cytotoxic effects which were synergistic in greater than 75% of the experimental combinations tested, based on isobologram analysis. The synergy in cell kill was confirmed by TUNEL assay. Flow cytometric analysis of the combination treatment

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indicated elimination of Tu138 cells from S and/or G<sub>2</sub>-M phases of the cell cycle. This synergistic combination may have therapeutic application in the treatment of head and neck cancer. The mechanism of synergistic action between these two agents may involve a commonality in signal transduction pathways induced by paclitaxel and ceramide.

Sphingolipids have been shown to be biologically active and have numerous regulatory effects on cell function including cell growth and differentiation. A number of inducers of sphingomyelin hydrolysis causing concomittant elevation of intracellular ceramide have been identified. These include TNF $\alpha$ , endotoxins, interferon  $\alpha$ , IL-1, Fas ligand, CD28, chemotherapeutic agents, heat and ionizing radiation (1, 2). The kinetics of endogeneous ceramide formation and accumulation appear to be complex and variable in different cell systems and with different inducers of sphingomyelin catabolism (3-6). It has recently been established that endogenously generated ceramide acts as a second messenger and induces apoptosis (7). Ceramide synthesis *de novo* has been implicated in lethal responses to several chemotherapeutic agents such as anthracyclines (8) and ara-C (9). Many recent studies have examined the effect of exogeneous ceramide on the induction of apoptosis in a variety of tumor cells. Ceramide has been shown in such cases to cause cell cycle arrest in several cell lines as well as apoptosis, cell senescence and terminal differentiation (10-13).

Paclitaxel is a chemotherapeutic drug with ability to block growing cells in the G<sub>2</sub>-M phase of the cell cycle (14). This is due to the fact that paclitaxel treated cells cannot undergo the microtubule depolymerization step to cytokinesis. In addition many paclitaxel mediated effects are known including bcl-2 phosphorylation (15) and the involvement of apoptosis via BAX, a bcl-2 dimerization product (16). Paclitaxel is currently being used in a variety of different cancers in clinical trials, alone and in combination with a

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variety of chemotherapeutic agents. (17-18) and has been shown to induce high rates of clinical responses in patients with squamous cell cancer of the head and neck (19). Neurotoxicity and myelosuppression seem to be the major limiting factors associated with paclitaxel treatment, hence there have been numerous studies combining other drug therapies with paclitaxel to increase its therapeutic efficacy. The purpose of the present studies was to examine if paclitaxel induced apoptosis can be enhanced by the addition of exogeneously added ceramide.

#### Materials and Methods

##### Tumor Cell Line:

Tu138, an adherent squamous carcinoma cell line of the head and neck was established at U T M D Anderson Cancer Center, Houston, TX (20, 21) and was generously provided by Dr. Gary Clayman's laboratory. Tu138 cells were maintained routinely in T-75 culture flasks (Falcon, Inc., NJ) at a plating cell density of  $0.1 \times 10^6$  per 75 sq. cm. surface area in complete D-MEM/F-12 culture medium (10 ml) containing 10% fetal bovine serum (FBS, Atlanta Biologicals, GA), 2 mM glutamine (Gibco, NY), 50 units/mL penicillin, 50 mg streptomycin (Gibco, NY) and 20 mM Hepes (Sigma, MO) in a 5% CO<sub>2</sub> atmosphere at 37°C. Tu138 cells were replenished with fresh complete culture medium twice weekly until confluence.

##### Treatment of Tu138 Tumor Cell Line with Drugs

For exposures to paclitaxel (taxol) and/or ceramide Tu138 cells were trypsinized in 0.25% trypsin-EDTA solution, washed twice in DMEM and plated in 96-well culture plates at a concentration of  $2 \times 10^4$  in a final volume of 0.2 ml in D-MEM/F-12 with 10% FBS and incubated in the presence or absence of different concentrations of paclitaxel (Taxol) (0-6000ng/ml; Bristol Myers Squibb, Inc., NJ) and/or ceramide (N-hexanoyl-D-sphingosine, 0-25 µg/ml; Sigma Chemicals, MO). At the indicated times (24 to 72 hours), cells were subjected

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to (a) tetrazolium-based dye assay for cell survival measurements, (b) flow cytometry analysis for cell cycle progression and (c) measurements of apoptosis by TUNEL assay.

5     MTT Assay

For the measurement of cellular cytotoxicity, 50  $\mu$ l of 0.2% solution of MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye (Sigma Chemicals, MO) was added to Tu138 cells after treatment with  
10     paclitaxel and/or ceramide and incubated at 37°C (22). At the end of an incubation period of 4 hours, culture plates were centrifuged at 300g for 2 minutes. After removal of the culture medium, 150  $\mu$ l of DMSO was added to solubilize MTT  
15     formazan crystals formed by cells undergoing coupled respiration and optical density was determined by the use of ELISA reader (Model EL 311, Biotek Inc.) at 540 nm.

Cell Cycle Measurements and TUNEL Assay

20     In concurrence with the MTT dye assay, Tu138 cells were plated in 6-well culture dishes at a concentration of  $2 \times 10^6$  in a volume of 4 ml in D-MEM/F-12 containing 10% FBS at 37°C and exposed to paclitaxel (600 ng/ml) and/or ceramide (25  
25      $\mu$ g/ml). At the end of either 24 or 48 hours, cells were trypsinized and washed with the culture medium. For cell cycle measurements, cells were mixed with 0.5 ml of 0.1% propidium iodide solution (New Concepts Scientific Ltd. ON) containing 0.1% sodium citrate and 0.1% NP-40. RNase (1  
30      $\mu$ g) was added to each sample and cells were incubated at 4°C for 30 minutes. At least  $1 \times 10^4$  cells were acquired on a precalibrated FACScan (Beckton Dickinson, CA) flow cytometer. For precalibration "DNA QC" (Becton Dickinson, CA) kit was utilized. The acquired cells were then subjected to cell  
35     cycle analysis by the use of "Modfit LT" software (Beckton Dickinson, CA). For measurements of apoptosis, In Situ Cell Death Detection Kit (Boehringer Mannheim, IN) followed by flow cytometry analysis was utilized.

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## Results

### Paclitaxel and Ceramide Induced Cytotoxicity as a Function of Time of Exposure

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The first set of experiments was directed to observe the cytotoxic effects of paclitaxel and ceramide at different concentrations separately on Tu138 cell line, as measured by MTT dye assay. As shown in Figure 6A, paclitaxel (Taxol) induced cytotoxicity was observed to be 37% and 64% at 600 and 6000 ng/ml respectively whereas ceramide induced cell effects after 3 days of culture resulted in 19% and 46% cytotoxicity at 12.5 and 25  $\mu$ g/ml concentrations respectively (Figure-6B). Multiple repeats (n=6) of these experiments indicated paclitaxel and ceramide induced ED<sub>50</sub> at concentrations of 1920 $\pm$ 1200 ng/ml and 22 $\pm$ 5  $\mu$ g/ml respectively.

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To evaluate whether the paclitaxel and ceramide combination enhances cellular cytotoxicity caused by either alone, Tu138 cells were incubated with and without paclitaxel (600 ng/ml) and ceramide (25  $\mu$ g/ml) simultaneously and separately as control. Cell viability was estimated using the MTT dye assay as a function of time every 24 hours over a period of 3 days. As shown in Figure 7, paclitaxel (Taxol) when added alone at a concentration of 600 ng/ml indicated cell cytotoxicity from 0.9% at 24 hours to a total of 21.8% by 72 hours. Ceramide also showed an increase in cytotoxicity from 32.7% at 24 hours to 54.5% at 72 hours. The combination of paclitaxel (600 ng/ml) and ceramide (25  $\mu$ g/ml) treatment showed an increase of approximately 68% in cell cytotoxicity over ceramide alone in a 3 day incubation period. The enhancement of cellular cytotoxicity was also evident in combination treatment at 24 (62.0% cell kill) hours with a maximum cell kill observed at 72 hours. From these results, the combination of both agents seems to indicate an enhancement over an additive effect with respect to a loss



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of electron transport and subsequent cell viability.

The results of 23 different observations of paclitaxel (600 ng/ml), ceramide (25  $\mu$ g/ml) and paclitaxel (600 ng/ml) plus ceramide (25  $\mu$ g/ml) combinations were then subjected to statistical analysis. The mean observed optical density (obtained after different exposures) and the standard deviation were analyzed over the confidence interval with 95% probability. The value of confidence range was compared by assuming a "Student t" distribution in the selected exposure conditions. The paired t-test for (n-1) degrees of freedom demonstrated that the results of paclitaxel and ceramide combination were significantly different with >95% probability than either paclitaxel or ceramide alone.

#### Synergistic Interaction Analysis by Isobologram

To confirm whether paclitaxel and ceramide interactions on Tu138 cell cytotoxicity was synergistic or additive a quantitative isobologram analysis (23, 24) of six independent experiments in triplicates was performed at various dilutions of paclitaxel (0-6000 ng/ml) and ceramide (0-25  $\mu$ g/ml) separately and simultaneously. A straight line joining points on x- and y- axes (Figure 8) represents ED<sub>50</sub> concentrations of paclitaxel (Taxol) and ceramide as determined from six independent experiments. Because of the non-linearity of the dose response curves with either paclitaxel or ceramide alone, a second line plot was generated by calculating ED<sub>10</sub> through ED<sub>90</sub> for either agent alone. The line plot represents ED<sub>10</sub> for paclitaxel combined with ED<sub>10</sub> of ceramide or paclitaxel ED<sub>30</sub> plus Ceramide ED<sub>20</sub> and so on. Figure 8 represents the isobologram using both the straight line approximation and curve generated from the experimental data to compare the effect of combined additions on synergy. The points to the left of the two line plots that fall within ED<sub>50</sub> concentrations indicate supra-additive properties of two agents tested in combination. The isobologram analysis indicated that six experiments

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represented as scatter plots are supra additive. The linear approximation consideration indicated 21 out of 23 experimental points to be synergistic. The experimental determination showed 18 of 23 points in the synergistic range.

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#### Paclitaxel and Ceramide Effects on Cell Cycle Distributions

Paclitaxel and ceramide effects have been implicated in cell cycle arrest and apoptosis. To examine whether the interactions of paclitaxel with ceramide would affect the kinetics of cell cycle progression, paclitaxel at 600 ng/ml and ceramide at 25  $\mu$ g/ml concentrations (at or near ED<sub>50</sub>'s) were utilized to measure the distribution of cells during growth by flow cytometer utilizing propidium iodide staining technique. Since it was difficult to obtain enough viable cells in a sample where combination treatment was utilized after 72 hour exposures, cell cycle measurements were made only at 24 and 48 hours. As Figure 9A-9H demonstrate paclitaxel (Taxol) was able to block 61.8% (24 hours) Figure 9C and 58.2% Figure 9G (48 hours) of T138 cells in the G<sub>2</sub>-M phase of the cell cycle in comparison to untreated control cells (7.4% at 24 hours and 6.0% at 48 hours). Ceramide added alone did not significantly affect either G<sub>0</sub>-G<sub>1</sub> or G<sub>2</sub>-M phase of the cell cycle progression similar to untreated controls (n=4) at either 24 or 48 hours.

In cultures treated simultaneously with paclitaxel (Taxol) and ceramide, a G<sub>2</sub>-M arrest of 45.6% (Figure 9D) and 28.4% (Figure 9H) was observed at both 24 and 48 hours respectively in comparison to paclitaxel (Taxol) treated cultures. This indicated a loss of 26.3% and 51.3% G<sub>2</sub>-M population of viable T138 cells due to the combined effect of paclitaxel and ceramide in comparison to paclitaxel alone at 24 and 48 hours respectively. A significant loss of S-phase population was also observed only at 48 hours. Taken in concurrence with the MTT results, these data indicate cell death occurring

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either in the G<sub>2</sub>-M phase or during the exit from the S-phase of the cell cycle.

5 Induction of Apoptosis by Paclitaxel Paclitaxel and Ceramide Combination

Since the MTT dye assay and the measurement of G<sub>2</sub>-M cell population cannot determine whether loss of cells is due either to necrosis or apoptosis, and since paclitaxel and ceramide when added alone have been shown to induce apoptosis by several researchers (25, 26), the next series of experiments were directed to examine the mode of cell kill induced by combined exposures of paclitaxel and ceramide at 24 and 48 hours by TUNEL assay. As demonstrated in Figure 10A-10H, exposures of paclitaxel (Taxol) (600 ng/ml) and ceramide (25 µg/ml) alone resulted in 18.4% and 9.7% apoptosis at 24 hours respectively whereas both added together induced 53.7% apoptosis indicating an approximately 3-fold increase in comparison to paclitaxel alone. At 48 hours, paclitaxel and ceramide individually induced a 54.8% and 13.6% apoptosis respectively when compared to observed apoptosis of 7.9% in untreated control. When the two agents were added together the percent apoptosis increased to 84.9%. In two separate experiments, paclitaxel and ceramide combinations showed 1.5-fold or greater apoptosis in comparison to paclitaxel alone at 24 and 48 hours.

Discussion

Paclitaxel has clearly been defined as an extremely important new agent in cancer chemotherapy. It has already demonstrated considerable antitumor activities against high staged and relatively resistant tumors of head and neck (18, 27-30). The present studies confirm that paclitaxel is able to arrest the head and neck carcinoma cell line Tul38 in the G<sub>2</sub>-M phase of the cell cycle. It is also demonstrated that paclitaxel alone is cytotoxic to head and neck carcinoma cells in vitro as demonstrated by the MTT assay (ED<sub>50</sub>:

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1920±1200 ng/ml). The large variation in ED<sub>50</sub> value may be attributed to asynchronous population of Tu138 cells during its growth. Such cell cycle sensitivity and cytotoxicity to paclitaxel has been demonstrated in ovarian, breast, lung and numerous other cell lines (31-34).

Exogeneous addition of ceramide has been shown to cause apoptosis in a variety of tumor cell lines (10, 35). In the current studies on solid tumors of head and neck cancer origin, ceramide mediated direct cytotoxicity of Tu138 cell is observed with an ED<sub>50</sub> of 22±5 µg/ml. In addition, ceramide was shown to induce apoptosis in Tu138 cells as measured by TUNEL assay. However, no significant effect of ceramide on the cell cycle progression of Tu138 cells was demonstrated. This is in contradiction to observed G<sub>0</sub>-G<sub>1</sub> cell block by ceramide in MOLT-4, a leukemic cell line (10).

The combined effect of paclitaxel and ceramide, two drugs with diverse activities, on neoplastic cell cycle progression was examined. It is postulated that if a chemotherapeutic drug such as paclitaxel were to enhance the accumulation of intracellular ceramide which has been suggested to be a mechanism of apoptotic cell death, then the addition of exogeneous ceramide may increase the threshold concentration leading to cellular cytolysis. While data on the necessary and sufficient concentration of intracellular ceramide to induce cytolysis is not available from these results, it is clear that these two agents work synergistically to induce apoptosis. This is confirmed by isobologram analysis demonstrating synergy in >78% of the tested experimental concentrations of paclitaxel and ceramide combined.

These present studies demonstrate that paclitaxel (600 ng/ml) and ceramide, when added separately, are able to kill Tu138 cells via apoptotic mechanisms within 24 hours of exposure. However, when the two agents were combined the observed cytotoxicity was greater than the addition of observed cytotoxicity by paclitaxel and ceramide separately indicating

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a synergistic interaction between these two agents. In addition, a significant reduction in the cell population in the G<sub>2</sub>-M phase of the cell cycle within 48 hours due to combined addition of paclitaxel and ceramide was evident from the measurements of cell cycle analysis. Taken together, the MTT dye and TUNEL assay results, seem to suggest that the loss of cell population is from either S or G<sub>2</sub>-M phase of the cell cycle.

The activation of a number of receptors including TNF $\alpha$ , interferon  $\gamma$ , and Fas signal an intracellular pathway that includes sphingomyelinases which act on membrane sphingomyelin and cause release of the lipid mediator ceramide (36-38). Ceramide may then cause the activation of down stream signal transduction pathways that in turn seem to initiate the final phase of apoptosis involving membrane blebbing, the breakdown of DNA and proteins, fragmentation of organelles, and packaging of cellular debris into apoptotic bodies (12, 39-40). Many chemotherapeutic agents such as doxorubicin have been shown to mediate apoptosis through the involvement of Fas receptor/ligand system (41), which has been questioned in a number of recent studies (42, 43). The engagement of FasR with paclitaxel is yet to be determined. Activation of apoptosis has also been shown to occur directly, through the activation of ceramide synthase with certain chemotherapeutic drugs such as daunorubicin (8). It has been shown that paclitaxel (Taxol) mediates its apoptotic effects via phosphorylation of bcl-2 (15) or the upregulation of BAX (16). Therefore, it is postulated that the mechanism whereby paclitaxel and ceramide mediate a synergistic effect may be via a commonality in downstream signal transduction pathways.

Paclitaxel has been evaluated in combination with cisplatin, doxorubicin and radiotherapy (44-46) in patients with encouraging results. It is important that new combinations be established with better efficacies and be evaluated for patient toxicities. The present results suggest that the

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addition of ceramide enhances the therapeutic potential of  
paclitaxel for treatment of head and neck carcinoma..

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#### 4th Series of Experiments

##### In vivo Studies Tu135 Cells

5 Ceramide, a sphingomyelin metabolite, provides the "death signal" for apoptosis in mammalian cells. Paclitaxel, an active chemotherapeutic agent, blocks cells in the G2/M phase of the cell cycle. It has been shown that paclitaxel and ceramide have synergistic cytotoxic effects on the leukemic T-cell line Jurkat (D. Myrick, 1999, in press). The  
10 interaction of paclitaxel and ceramide in a variety of solid tumor cancer cell lines is further explored herein to determine whether there is similar synergism in inducing cytotoxicity and apoptosis.

#### 15 MATERIALS AND METHODS

Neoplastic human cell lines included Prostate (LnCaP), Colon (HT29), Pancreatic Cancer (RWP-2) and head and neck squamous carcinoma (TU138) which were maintained in RPMI-1640  
20 containing 10% fetal bovine serum. C6-Ceramide (Sigma Chemicals) initially dissolved in ethanol and paclitaxel (Taxol) (Bristol-Myers, Squibb, Inc.) were diluted in RPMI. Each neoplastic cell line at  $25 \times 10^3$  was incubated at 37°C in the presence and absence of Paclitaxel with varying  
25 concentrations of Ceramide and were subjected to the MTT dye assay for surviving cell fractions at 72 hrs.

#### IN VIVO STUDIES

30 TU138 cells were implanted subcutaneously in nude mice Balb/c NU/NU which were treated beginning on day 4 with thrice weekly injections of Paclitaxel 120  $\mu$ g/0.1ml, Ceramide, 500  $\mu$ g in 0.2 ml, or the combination and control vehicle, and were observed over 6 weeks.

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#### RESULTS

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Paclitaxel induced cytotoxicity in most tumor cell lines tested with an ID50 of 6.0-60.0 ng/ml. Ceramide was equally cytotoxic at calculated ID50 of approximately 30.0-40.0  $\mu$ g/ml. Combination of the two enhanced cellular cytotoxicity in a synergistic manner (as indicated by the 50% isobologram).

**TABLE 2**

%Growth Inhibition of Human Cancer Cell Lines by Paclitaxel (Taxol) and Ceramide Alone and in Combination

CELL LINE	PACLITAXEL (600 ng/ml)	CERAMIDE (25 $\mu$ g/ml)	PACLITAXEL (600 ng/ml) plus CERAMIDE (25 $\mu$ g/ml)
HT29	16%	61%	81%
Jurkat	55%	8%	78%
LnCap	13%	25%	66%
PC-3	16%	8%	51%
RWP-2	2%	6%	75%
TU138	10%	28%	66%

Tumor growth of TU138 was significantly inhibited by combination of Paclitaxel and Ceramide (Figs. 11 & 12)

#### DISCUSSION

Ceramide enhances Paclitaxel-mediated cytotoxicity in several tumor cell lines and augments the anti-tumor effect of paclitaxel on one of the lines (TU 138) in an in vivo nude mouse model. The mechanism is not yet defined but in part appears related to cell cycle switching by Paclitaxel (G2/M cell cycle block) although other considerations related to apoptosis are under study (bcl-2 and BAX system, changes in intracellular ceramide and Fas ligand mediated events).

### 5th Series of Experiments

#### Treatment of Breast Cancer with Chemotherapeutic Agents and Ceramide

5

10 Cancer of the breast continues to be the most common cause  
of death in middle aged women. Both its annual incidence  
and mortality in western countries are on the increase and  
the majority of women who develop breast cancer ultimately  
15 die of recurrent or metastatic disease. It is important to  
utilize new drugs in maximally efficient therapeutic  
strategies to slow or eliminate the disease progression.  
This invention provides an innovative and explorative method  
of treatment using the lethal combination of diverse agents,  
20 paclitaxel and a naturally occurring ceramide. This  
invention concurrently provides the therapeutic basis of  
paclitaxel action on breast tumor cell in vitro leading to  
better and less toxic therapeutic strategies. The  
explorative method has evolved from different experimental  
25 protocols. Specific aims of the present study include  
demonstrating most effective conditions of paclitaxel and of  
ceramide (a) to affect growth arrest leading to subsequent  
synergistic cytotoxic effects in combined protocols; (b) to  
establish if liposome formulation can enhance the synergistic  
30 action of both agents. It will also establish if both agents  
can be formulated together in liposome to enhance their  
effects.

35 Cancer of the breast continues to be the most common cause  
of death in middle aged women. Both its annual incidence and  
mortality in western countries are on the increase and the  
majority of women who develop breast cancer ultimately die  
of recurrent or metastatic disease. Despite a considerable  
research effort and controlled clinical trials, the  
management of breast carcinoma is as confused and shows  
partial success until drug resistant tumors begin to grow.  
It is important to utilize new drugs in maximally efficient  
therapeutic strategies to slow or eliminate the disease

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progression.

This innovative and explorative study uses the most lethal combination of two diverse agents, paclitaxel and a naturally occurring compound termed ceramide. Concurrently, the in vivo application of these two agents in an animal model utilizing human breast carcinoma cells is examined. This explorative method has evolved from the application of these two agents first, in in vitro studies of a variety of neoplastic cell lines including leukemic, prostate, pancreatic and squamous cell carcinoma and second, in an in vivo study of a squamous carcinoma cell line in nude mice.

These preliminary studies have shown that the combination of paclitaxel and ceramide given at the time of the initiation of cultures in vitro induces apoptosis in a variety of cell lines synergistically as measured by either flow cytometry, Wright cell analysis or by a TUNEL assay. The synergistic interaction was also confirmed by isobologram analysis. These studies also demonstrated that the apoptotic cells were derived from either S or the G<sub>2</sub>-M phase of the cell cycle. The studies on the mechanisms of action of these agents when added separately have identified few common pathways leading to apoptosis. It is hypothesized that paclitaxel or ceramide exposures will lower the required threshold of each agent, thereby reducing patient toxicity.

Just as combination chemotherapy is superior to single agent chemotherapy in advanced disease, it is plausible that better drug combinations will lead to improved adjuvant therapy results. Improved understanding of drug-cell interactions will not only expand present knowledge of therapy but also provide some indication of the design for future trials.

Although significant progress has been made in the management of breast carcinoma, improved treatment strategies are urgently needed for both locally advanced and metastatic



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breast carcinoma. Paclitaxel is a new agent with important cytotoxic activity in a variety of tumor cells and has demonstrated radiation sensitizing ability in select tumor cell models. The role of paclitaxel in the treatment of breast carcinoma is beginning to be established although its cellular mechanisms of action are still being defined. It is critical, however, to design adjuvant, neo adjuvant therapy for the treatment of recurrent disease.

#### 10 Tumor Cell Growth and Drug Selection

The most important feature of malignancies is the clonal origin of tumor cells and their retention of proliferative capacity (1). The principle of therapy with cytotoxic drugs relies on the selective inhibition/lysis of neoplastic cells and not the normal cells or tissues. The basis for most chemotherapeutic drug action is the inhibition of DNA, RNA and/or protein synthesis. Since the growth of tumors is usually described by Gompertzian kinetics (2, 3), the effectiveness of chemotherapeutic drugs is determined by tumor growth fraction, doubling time and size. Furthermore, the application of chemotherapy, as outlined by the Goldie-Coldman (4) model, suggests that cells will mutate to develop drug resistance based on the inverse relationship between cure and cell number and is independent of the neoplastic cell growth kinetics. This model provides the basis for (a) early initiation of treatment when tumor burden or fraction and the likelihood of drug resistance is small and (b) effective treatment utilizing a combination of drugs to lower side effects and toxicities to achieve maximal neoplastic cell kill.

The specific drug selection in a given protocol is usually based on the mechanism of action of the drug in question, its effects on the cell cycle, its toxicity and its effectiveness against a given tumor. Paclitaxel (Taxol) is another antineoplastic agent isolated from the *Taxus brevifolia*, more commonly known as the Western Yew (5). The proposed

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mechanism whereby paclitaxel induces cytotoxicity is the stabilization of polymerization of tubulin monomers and prevention of depolymerization. This leads to the arrest of the paclitaxel responsive cells in the G<sub>2</sub>-M phase of the cell cycle. In contrast to Vincristine which affects the assembly of microtubules, paclitaxel blocks the depolymerization of assembled microtubules (6), thereby causing arrest of growing cells in the mitotic phase of the cell cycle. The formation of multidrug resistant phenotype with the overproduction of P-glycoprotein has been shown to be a consequence of paclitaxel exposure (7) suggesting the need for developing treatment strategies which minimize the development of resistance. Although a variety of research projects and clinical trials have recently sought to determine the antineoplastic action of paclitaxel in relation to breast carcinomas, its direct cellular (cell cycle progression) and molecular effects are only beginning to be elucidated.

#### Breast Cancer: Paclitaxel in Clinical Therapy

Paclitaxel as a single agent (n=25, 250 mg/m<sup>2</sup>, 24 hour infusion every 21 day interval) has been evaluated in clinical trials of recurrent breast cancer with a complete and partial response rates of 12% and 44% respectively (8). The dose related toxicity, however, was severe. Interestingly, in a therapeutic regimen of paclitaxel with doxorubicin it was observed that patients had impairment in the elimination of doxorubicin if paclitaxel was administered first suggesting a need for drug sequencing studies in a combination protocol. In Eastern Cooperative Oncology Group (ECOG) studies (9) the maximally tolerated doses of doxorubicin (60 mg/m<sup>2</sup>) and paclitaxel (175 mg/m<sup>2</sup>) are given as a 24 hour continuous infusion in different combinations over a period of 3 weeks. In a separate study of chemotherapy-naive metastatic breast cancer, a 3 hour infusion of paclitaxel (90 mg/m<sup>2</sup>) is followed by a 60 mg/m<sup>2</sup> cisplatin every 2 weeks (10). This protocol allows for an increase in the dose of paclitaxel up to 130 mg/m<sup>2</sup> without

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changing the cisplatin dose. While severe toxicity is observed, the results of this study demonstrate 25% complete and 69% partial response rates with an overall response rate of 94%. In another combination therapeutic protocol for recurrent breast carcinoma (n=40), paclitaxel (135 mg/m<sup>2</sup>) has been administered with cyclophosphamide (750 mg/m<sup>2</sup>) in various combinations with the following results (11): partial response: 4, no change: 18 and progressive disease: 18. While the efficacy of paclitaxel as a single agent is yet to be established, its combination with different agents has shown promising results in selected patients.

Breast Cancer: In Vitro and In Vivo Animal Studies with Paclitaxel

Many in vitro cellular studies and in vivo animal studies with human breast carcinoma cells have sought to examine the effect of various cytotoxic agents with paclitaxel. These cytotoxic agents include etoposide, vinorelbine, cis-platin, vinblastin and tamoxifen. The studies with etoposide in combination with paclitaxel demonstrated optimal effect on MCF-7, a breast carcinoma cell line to be dependent on the delivery schedule of the drugs in a sequential manner (12). Paclitaxel induced growth inhibition of MCF-7 in the presence of cis-platin demonstrated merely additive effects (13). Interestingly, while both vinorelbine and paclitaxel bind to distinct sites on tubulin with opposing effects on microtubules they demonstrated synergistic effects on MCF-7 cell cytotoxicity when administered concurrently. Paclitaxel has been demonstrated to be efficacious on hormone refractory breast cancer cells (14), tumor cells with disruptions in p53 (15) or cells with overexpression of HER-2 gene (16).

Additionally, many recent studies have examined (a) the differential effects of drugs or agents on cell cycle progressions. In these studies, panel of cells including breast carcinoma were combined with adenovirus-mediated gene

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therapy ( $G_0$ - $G_1$  blocker) in combination with either simultaneous or post paclitaxel ( $G_2$ -M blocker) treatment (17). While no interactive antagonistic activity was observed, the combination had either synergistic or additive effect. Interestingly, the relative concentration of each agent determined the dominant response in terms of cell cycle progression and cell death; (b) the activation of molecular pathways leading to apoptosis with the help of radioimmunotherapy. These studies demonstrated that timing and sequencing of paclitaxel and 90Y-DOTA-peptide-ChL6 antibody (90Y-ChL6) is critical in combined modality for maximal effectiveness in human breast carcinoma cells (HBT 3477) in nude mice (18); and (c) the inhibition of angiogenesis by paclitaxel in addition to Vincristin, colchicin and 2-methoxyestradiol (19).

It is clear the review of literature on paclitaxel presented here indicates the following: (a) paclitaxel in combination with other drugs is more effective than as a single agent, (b) the definition of the mode of addition of different agents in a combination protocol is essential to achieve maximum therapeutic benefit, (c) the mechanism of action for the induction of apoptosis may be dependent on the concentration of available paclitaxel and (d) the sequential addition of low dose over an extended period of time may be more effective than one big bolus of paclitaxel. These studies also suggest that the discovery of a new agent, ceramide, that complements and amplifies the action of paclitaxel is important consideration in therapeutic strategies to eventually eliminate the abnormal growth of tumor cells.

#### Ceramide and Breast Cancer

Sphingolipids have been shown to be biologically active and have numerous regulatory effects on cell function including cell growth and differentiation. A number of inducers of

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sphingomyelin hydrolysis causing concomittant elevation of intracellular ceramide have been identified. These include TNF $\alpha$ , endotoxins, interferon alpha, IL-1, Fas ligand, chemotherapeutic agents, heat and ionizing radiation (20).

5 Ceramide synthesis de novo has been implicated in lethal responses to several chemotherapeutic agents such as anthracyclins (21) and ara-C (22). Many recent studies have examined the effect of exogeneous ceramide on the induction of apoptosis in a variety of tumor cells. Ceramide has been

10 shown in such cases to cause cell cycle arrest in several cell lines as well as apoptosis, cell senescence and terminal differentiation (23-26). The role of ceramide induced cytotoxicity in breast carcinoma is reported by many laboratories. These studies have examined the effect of cell

15 permeable C6-ceramide to cause apoptosis in TNF $\alpha$  resistant MCF-7 cell line and increase in intracellular ceramide as a consequence of sphingomyelin hydrolysis due to the addition of TNF $\alpha$  on sensitive MCF-7 cells (27). Cell permeable C2-ceramide was shown to cause apoptosis in another human breast

20 cancer cell line, Hs578T which was enhanced by the addition of insulin-like growth factor binding protein-3 (28). Interestingly, suramin-induced cell death in human breast, prostate and neuron like cell lines have been shown to have elevated levels of intracellular ceramide prior to apoptosis

25 (29).

#### Mechanisms of Action in Paclitaxel or Ceramide Induced Apoptosis

30 There are many proposed mechanisms of action for paclitaxel induced cell death. These include the phosphorylation of bcl-2 (30) in prostate cancer cells, transient activation of JNK pathway (31) in leukemic cells and sensitization and activation of FasR on cell surface (1st series of

35 experiments). Similarly, multitudes of apoptotic pathways leading to the activation of bcl-2/BAX, JNK/SAPK, FasR/FasL, etc. have been proposed as a consequence to ceramide addition

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in a variety of cellular models (32). It should be pointed out that no studies have recorded the combined effects of paclitaxel and ceramide on many of these proposed pathways.

5 Preliminary Studies: Paclitaxel and Ceramide Action In Vitro

The present studies (1st series of experiments) with a human leukemic T-cell line, Jurkat have demonstrated that the addition of C6-ceramide enhanced paclitaxel-mediated  
10 apoptosis in a synergistic manner. These studies also established that the apoptotic population of Jurkat cells was from either S or G<sub>2</sub>-M phase of the cell cycle. In these studies, the apoptosis was measured by Wright stain, TUNEL assay and flow cytometric evaluations.

15 Paclitaxel and cell permeable C6-ceramide mediated synergy for observed apoptosis was also evident in a squamous carcinoma cell line, TU-138. The evaluation of apoptosis was carried out by TUNEL assay. Flow cytometric studies  
20 confirmed the earlier observations with Jurkat cells that the loss of cells to apoptosis was derived from either S or G<sub>2</sub>-M phase of the cell cycle. The synergistic response of paclitaxel and ceramide was confirmed by the isobologram analysis and its significance was established by student's  
25 T-test.

It was also observed that paclitaxel and C6-ceramide induced synergistic growth inhibition in a variety of tumor cell lines including prostate (hormone sensitive LnCaP and hormone  
30 refractory PC-3), pancreatic (RWP-2) and breast (MCF-7) carcinoma cell lines.

In addition, the studies with breast carcinoma cell line, MCF-7 have suggested that the concentration of paclitaxel  
35 added at the onset of cultures is critical to the progression of cell cycle. At lower than ID<sub>50</sub> paclitaxel concentrations (0.6 - 6.0 ng/ml), it was more efficacious, blocking greater than 55% of cells in to G<sub>2</sub>/M phase of the cell cycle.

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Interestingly, at higher than  $ID_{50}$  concentrations, paclitaxel clearly affected the movement of cells from  $G_1$  phase of the cell cycle reducing the number of cells into  $G_2/M$  phase to 38%.

5

Preliminary Studies: Paclitaxel and Ceramide Action In Vivo

To corroborate the in vitro findings of synergistic action of paclitaxel and ceramide, TU-138 cells were planted subcutaneously in nude mice at day 0. The treatment with paclitaxel ( $120\mu\text{g}$  in 0.1 mL) and ceramide ( $500\mu\text{g}$  in 0.2 mL) was begun on day 4 with thrice weekly injections administered subcutaneously near the site of the tumor implantation on squamous cell carcinoma cell line TU-138 for a period of 5 weeks. The following control groups were run: vehicle only, paclitaxel alone and ceramide alone. The size of the tumor was measured once a week and continued for 6 weeks (Figure 11). All groups of mice were sacrificed on day 45 and the excised tumor was weighed (Figure 12). The data presented are preliminary, but nevertheless indicate that TU-138 cell-tumor grew aggressively in control and paclitaxel treated mice almost equally. The group with only ceramide injections demonstrate a slight decrease in tumor size at week 5. The group with both paclitaxel and ceramide showed a decline of 79% in tumor size in comparison to control group.

The significant reduction (88% in paclitaxel + ceramide in contrast to control only) in tumor weight corroborated these findings as shown in Figure 12. Interestingly, the ceramide only group did demonstrate a substantial reduction in tumor weight. The data is currently being evaluated statistically. The excised tumors are currently being subjected for histopathological studies to determine tumor cell morphology and cellular apoptosis.

35

The existing protocols of combination chemotherapy utilizing paclitaxel are only partially effective. Therapeutic

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strategies that involve ceramide *in vivo* in breast carcinoma have not been reported yet.

5 Paclitaxel induces transient growth arrest of breast cancer cells into the G2-M phase of the cell cycle. A simultaneous or progressive addition of ceramide, a G<sub>0</sub>-G<sub>1</sub> blocker, will result in greater apoptosis either by targeting those cells that escaped paclitaxel effects or by activating complementary apoptotic pathways. This will result in  
10 increased effects of paclitaxel at concentrations lower than those currently employed in clinical protocols.

#### Technical Objectives

15 The long-term objectives of this study include determination of the most efficacious clinical and therapeutic basis of paclitaxel effects on breast cancer cells. These studies focus on (a) examinations of the kinetics of the combined effects of paclitaxel and ceramide in cell growth inhibition  
20 and lysis *in vitro* and (b) corroboration of the *in vitro* studies in an *in vivo* nude mouse model utilizing breast carcinoma cell line. The specific aims of the studies are set forth below.

25 Evaluation of paclitaxel and ceramide in a combination therapy utilizing a nude mouse model bearing human breast tumor: Paclitaxel as a single agent has been shown to negatively modulate the growth of breast carcinoma in the clinic and also in the laboratory studies. Cell-permeable  
30 exogenously added ceramide has also been shown to induce apoptosis in a variety of cancer cell lines *in vitro* including breast carcinoma. The studies combine these two agents with diverse mechanism of actions to enhance the cytotoxic effects of each other. This innovative approach is  
35 based on the rationale that unaffected cells by one agent will be targeted by the other agent as a direct effect. At the same time these two agents will amplify the converging



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point(s) in an otherwise enhanced pathway toward apoptosis resulting in a synergistic interaction. Such synergistic action has been observed with paclitaxel and ceramide in a squamous carcinoma cells grown in vivo in nude mice in the laboratory.

Establish the necessary and sufficient conditions for the growth inhibition and cellular cytotoxicity effects of paclitaxel and ceramide when added simultaneously or in sequence followed by the determination of apoptosis of the cells of breast carcinoma: The studies focus on the kinetics of the addition of paclitaxel and/or ceramide and their effect on cell cycle progression of breast cancer cells. The studies also investigate apoptosis by a variety of available methods.

#### Experimental Design and Methods:

##### **Breast Cancer and Drug Delivery**

Combination drug delivery is an essential component of the inhibition of tumor cell telesis (programmed cell growth) and eventual apoptosis (programmed cell death). Cancer treatment with paclitaxel has been coupled with different therapeutic modalities which include radiation and/or chemotherapeutic agents as described earlier. For in vitro studies many different breast cancer cell lines have been utilized to establish the effects of combination of drugs. Nude mouse model with implanted human breast cancer cells have been utilized in a number of studies. In these studies drugs have been delivered in a variety of ways including intraperitoneal, intravenous or subcutaneous injections. The vehicle for drug delivery is also an essential component as it affects drug stability, toxicity and pharmacokinetics. Paclitaxel has been delivered in cremophore or liposomes in many animal studies (33). Sphingolipids have been delivered in vivo via liposome preparations (34).

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These preliminary studies with squamous cell carcinoma in nude mice (Balb/c strain, Taconic Farms, NY) paclitaxel and ceramide were delivered in very small amounts in cremophore and alcohol respectively. In the first phase of these studies the paclitaxel is delivered in cremophore and C6-ceramide in alcohol solution diluted with culture medium. These studies include all appropriate vehicle controls (medium, alcohol and/or cremophore alone) in appropriate concentrations. The experimental and control group also includes paclitaxel, ceramide and a combination of paclitaxel and ceramide. These agents are delivered on day 4 after the implantation of MCF-7 (breast cancer cell line) cells in nude mice. Appropriate drugs are injected subcutaneously three times weekly and the growth of tumor is followed for approximately 6-8 weeks. This time period is derived from preliminary studies on head and neck cancer cells in nude mice. These studies are initially done in various groups (including controls all done at the same time) with 5 mice each. This set of studies is repeated at least 3 times and the data subjected to statistical analysis to obtain the confidence limits.

As indicated earlier liposome preparations of paclitaxel have been as efficacious or better than standard preparations in cremophore. Also, liposome preparations of sphingolipids have been shown to have greater effect because of its stability than free sphingolipids (34). Therefore, C6-ceramide may be prepared in liposomes as indicated in the methods section. The experimental design with liposomal formulations includes appropriate controls including liposome preparations without ceramide and/or paclitaxel. Liposome formulations may be administered intraperitoneally as well.

A set of in vitro experiments is performed to assess the effect of liposome formulations on MCF-7 with appropriate controls. In these experiments, cells are subjected simultaneously or sequentially with paclitaxel and ceramide. If results indicate simultaneous treatment to be better than

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sequential treatment then a single liposome formulation with paclitaxel and ceramide combined will be prepared and tested again with its appropriate controls as indicated earlier. A minimum of 15 nude mice in three separate sets of experiments are tested in experimental protocols. Based on studies with squamous carcinoma cells that a sum total of these experiments provide enough samples to be tested for significance by statistical analysis.

For the evaluation of the combined effects of paclitaxel and ceramide *in vivo* the following measurements are performed: (1) tumor growth in millimeters, (2) excised tumor weight at the completion of experiments, (3) histopathology of excised tumors and (4) the identification of apoptotic cells using TUNEL method. The measurement of tumor growth and weight is a routine procedure and is performed by a trained animal technician. The identification of apoptotic cells by TUNEL assay is also a routine procedure in the laboratory and is performed on a FACScan flow cytometer which is part of the core facility available to all investigators. The histopathology of the excised tumors is then performed.

The evaluation of the combined effects of paclitaxel and ceramide on MCF-7 cells *in vitro* involve the measurement of cell growth by a tetrazolium (MTT) dye assay (a routine procedure), apoptosis by TUNEL assay and cell cycle progression by flow cytometry (also a routine procedure).

The liposome formulations are prepared by a standard lipid film procedure. Briefly, paclitaxel, cardiolipin, cholesterol and phosphatidylcholine are dissolved in chloroform-methanol mixture as described (33, 34). The solvents are evaporated in a rotary evaporator under vacuum. The hydrated film is dispersed by vigorous mixing. The small unilamellar liposomes are then obtained by sonication. The amount of drug entrapped is determined by standard reverse-phase high pressure liquid chromatography. The stability of these preparations is determined first by

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regular in vitro cell growth inhibition assays on MCF-7 cells in comparison to paclitaxel in cremophore and ceramide in alcohol solutions. The liposome formulations are utilized based on their stability assays. It is anticipated that these preparations will be stable for a period of 4 weeks at 4°C. The usual problem with the liposome formulations is the fusion of the final product with time. In such situations the formulated products are sized through an extruder using 50 nm NUCLEOPORE membrane.

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### Sixth Series of Experiments

#### In Vivo Studies on the Effects of Paclitaxel and Ceramide

Currently there are no reported studies on the combined effects of paclitaxel and ceramide *in vivo* on any cancer cell model. Initial experimentation to corroborate *in vitro* findings of synergistic action of paclitaxel and ceramide has utilized Tul38 cells (a solid tumor of the head and neck cancer origin) that were planted subcutaneously (sc) on nude mice (experimental group, 5 mice/group) at day 0. The treatment with paclitaxel (120 $\mu$ g in 0.1ml) and ceramide (500 $\mu$ g in 0.2ml) was begun on day 4 with three weekly injections administered subcutaneously near the site of the tumor implantation for a period of 5 weeks. The following control groups (5 mice per group) were run: vehicle only, paclitaxel alone and ceramide alone. The size of the tumor was measured once a week and continued for 6 weeks (Figure 13). All mice were sacrificed on day 45 and the excised tumor was weighed (Figure 14). The data presented indicate that Tul38 cell-tumor grew aggressively in control and paclitaxel treated mice almost equally. The group with only ceramide injections demonstrates a slight decrease in tumor size at week 5. The group with both paclitaxel and ceramide showed a decline of 79% in tumor size in comparison to the control group. The significant reduction (88% in paclitaxel + ceramide in contrast to control only) in tumor weight corroborated these findings as shown in Figure 13. Interestingly, the ceramide only group did demonstrate a substantial reduction in tumor weight.

In a separate experiment, nude mice were implanted with Tul38 cells followed by treatment with paclitaxel (120 $\mu$ g) and ceramide (500 $\mu$ g) as indicated above. Paclitaxel and/or ceramide were delivered in cremophore as a combination. The vehicle control consisted of cremophore only at the concentration utilized in the combination treatment. The results presented in Table 3 show that the concentration of paclitaxel utilized reduces tumor growth by 11.1%, whereas,

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ceramide mediated reduction in tumor size is 44.7% and that the combination treatment reduces tumor size significantly by 83.3% similar to studies presented in Figure 13. The data was subjected to group t-test (on Statview software) and p values are provided in the table legend.

The next sets of experiments were directed at the drug delivery methods whereby paclitaxel and ceramide combination was given intraperitoneally at the same concentrations as described for sc injections. The results of these studies are shown in Figure 15. The analysis of these results indicate that the combination protocol of treatment with paclitaxel and ceramide given intraperitoneally reduced the tumor burden by 60% within 4 weeks of drug administration in comparison to control mice with vehicle injections only. It should be noted that paclitaxel alone treatment reduced tumor size by 29% in comparison to control alone; whereas, ceramide alone had no significant effect indicating synergistic interactions in tumor cytotoxicity by combined treatment protocol.

In summary, results of the sixth series of experiments indicate that (a) both modes of delivery, intraperitoneal and subcutaneous, were effective in the combination treatment to reduce tumor burden greater than the single agents alone, (b) there is no toxicity associated with cremophore or ceramide delivered in cremophore and (c) this unique combination is effective in preclinical *in vivo* studies.

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TABLE 3

Growth of Human Tu138 Cells in Nude Mice:  
Delivery of Paclitaxel and Ceramide in Cremophore

	<u>Experimental Group</u>	<u>Tumor Size (mm<sup>2</sup>) at Week 5</u>
	Vehicle Control	198.0 + 124.4 (n=5)
10	Paclitaxel	176.0 + 90.3 (n=5)
	Ceramide	109.5 + 14.1 (n=6)
	Paclitaxel + Ceramide	33.0 + 31.8 (n=6)

In a separate experiment nude mice were implanted with  $1-2 \times 10^6$  Tu138 cells. The treatment with paclitaxel (120 $\mu$ g) and ceramide (500 $\mu$ g) was begun on day 4 with thrice weekly injections administered subcutaneously near the site of the tumor cell implantation for a period of 5 weeks. The size of the tumor was measured once a week and continued for the duration. Paclitaxel and ceramide in combination preparations were delivered in cremophore. The vehicle control consisted of cremophore only at the concentration utilized in the combination treatment. The data was subjected to group t-test (on Statview software) with the following analysis:

	<u>Group Comparisons</u>	<u>p Value</u>
	Vehicle Control - Combination Treatment	0.005 < p $\leq$ 0.01
20	Paclitaxel alone - Combination Treatment	0.005 < p $\leq$ 0.005
	Ceramide alone - Combination Treatment	p $\leq$ 0.0005
	Paclitaxel alone - Ceramide alone	0.05 < p $\leq$ 0.1

25

30

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What is claimed is:

1. A method for increasing apoptosis in tumor cells comprising contacting the tumor cells with:
  - a) an effective amount of at least one antitumor chemotherapeutic agent and
  - b) an effective amount of a ceramide, sequentially or concomitantly, wherein the apoptosis induced by the combination of the antitumor chemotherapeutic agent and the ceramide is greater than the apoptosis induced by contact of the tumor cells with either the antitumor chemotherapeutic agent alone or the ceramide alone, thereby increasing apoptosis in tumor cells.
2. A method of decreasing a size of a tumor comprising contacting the tumor with:
  - a) an effective amount of at least one antitumor chemotherapeutic agent and
  - b) an effective amount of a ceramide, sequentially or concomitantly, wherein the induced decrease in size of the tumor by the combination of the antitumor chemotherapeutic agent and the ceramide is greater than the decrease in size of a tumor after contacting the tumor with either the antitumor chemotherapeutic agent alone or the ceramide alone, thereby decreasing the size of the tumor.
3. The method according to either claim 1 or 2, wherein the tumor cells are or the tumor is composed of cancer cells selected from the group consisting of leukemic cells, prostate cancer cells, pancreatic cancer cells and squamous cell carcinoma cells, breast carcinoma cells, melanoma cells, basal cell carcinoma cells, neuroblastoma cells, glioblastoma multiforme cells, myeloid leukemic cells, colon carcinoma cells, endometrial carcinoma cells, lung carcinoma cells, ovarian carcinoma cells, cervical carcinoma cells, osteosarcoma cells and lymphoma cells.

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4. A method according to either claim 1 or 2, wherein the tumor cells or the tumor are contacted first with at least one antitumor chemotherapeutic agent and subsequently contacted with the ceramide.  
5
5. A method according to either claim 1 or 2 wherein the tumor cells or the tumor are present in a subject.
6. The method according to either claim 1 or 2, wherein the ceramide is selected from a C2-ceramide, C6-ceramide, C8-ceramide and a higher order ceramide.  
10
7. The method according to either claim 1 or 2, wherein the antitumor chemotherapeutic agent is selected from the group consisting of paclitaxel, doxorubicin, cis-platin, cyclophosphamide, etoposide, vinorelbine, vinblastin, tamoxifen, colchinin, and 2-methoxyestradiol.  
15
8. The method according to either claim 1 or 2, wherein the contacting with the antitumor chemotherapeutic agent is effected by cremophore delivery or liposome-mediated delivery and the contacting with the ceramide is effected by cremophore delivery, alcohol-mediated delivery or liposome-mediated delivery.  
20  
25
9. The method according to either claim 1 or 2, wherein the contacting with the antitumor chemotherapeutic agent and with the ceramide is effected by an administration route selected from the group consisting of intravenous, intraperitoneal, intrathecal, intralymphatical, intramuscular, intralesional, parenteral, epidural, or subcutaneous administration; by infusion, by aerosol delivery; or by topical, oral, nasal, anal, ocular or otic delivery.  
30  
35

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- 5           10.       A pharmaceutical composition comprising at least one antitumor chemotherapeutic agent in an amount effective to induce apoptosis of tumor cells and a ceramide in an amount effective to induce apoptosis of tumor cells and a pharmaceutically acceptable carrier.
- 10           11.       A method for treating cancer in a subject comprising administering to the subject an effective amount of at least one antitumor chemotherapeutic agent and an effective amount of at least one ceramide, sequentially or concomitantly.
- 15           12.       The method according to claim 11, wherein at least one antitumor chemotherapeutic agent and subsequently at least one ceramide is administered to the subject.
- 20           13.       The method according to claim 11, wherein at least one ceramide and subsequently at least one antitumor chemotherapeutic agent is administered to the subject.
- 25           14.       A method according to claim 11, wherein the antitumor chemotherapeutic agent is paclitaxel and the ceramide is C6-ceramide.
- 30           15.       A method according to claim 11, wherein the ceramide is a C2-ceramide, C6-ceramide, C8-ceramide or a higher order ceramide.
- 35

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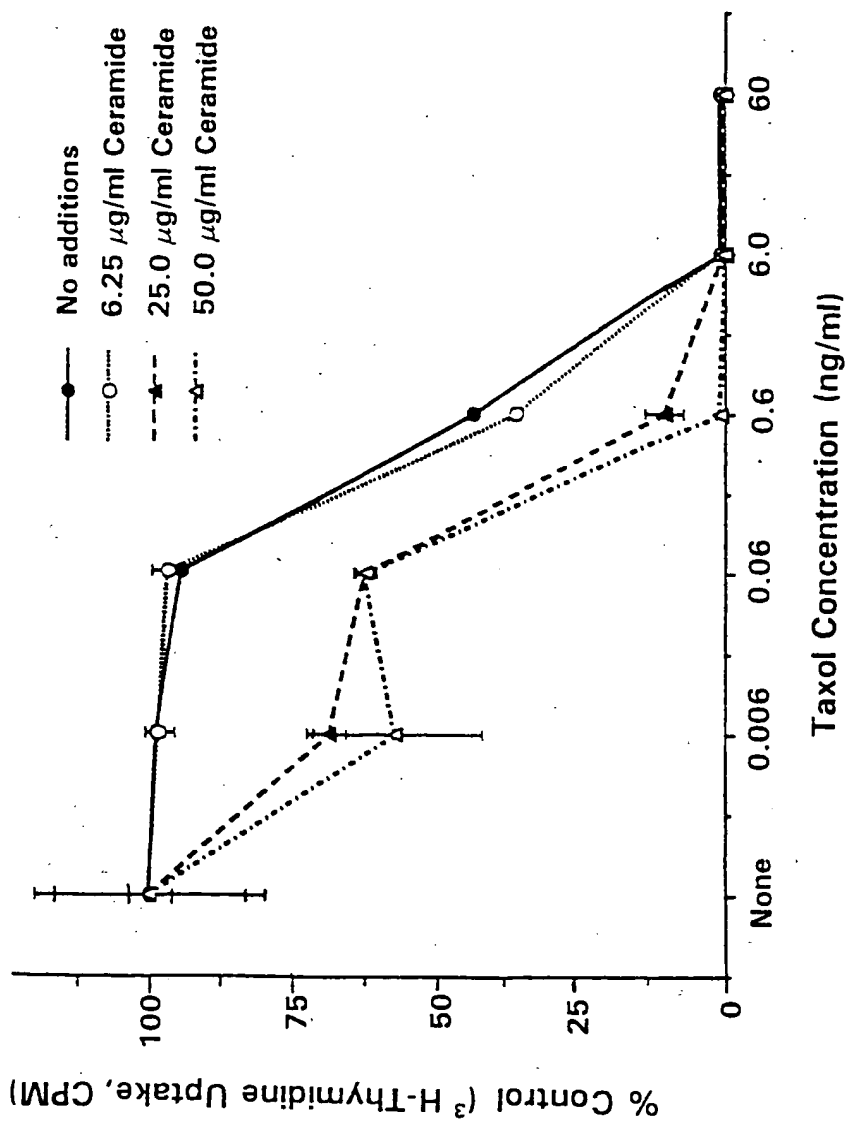


FIG. 1

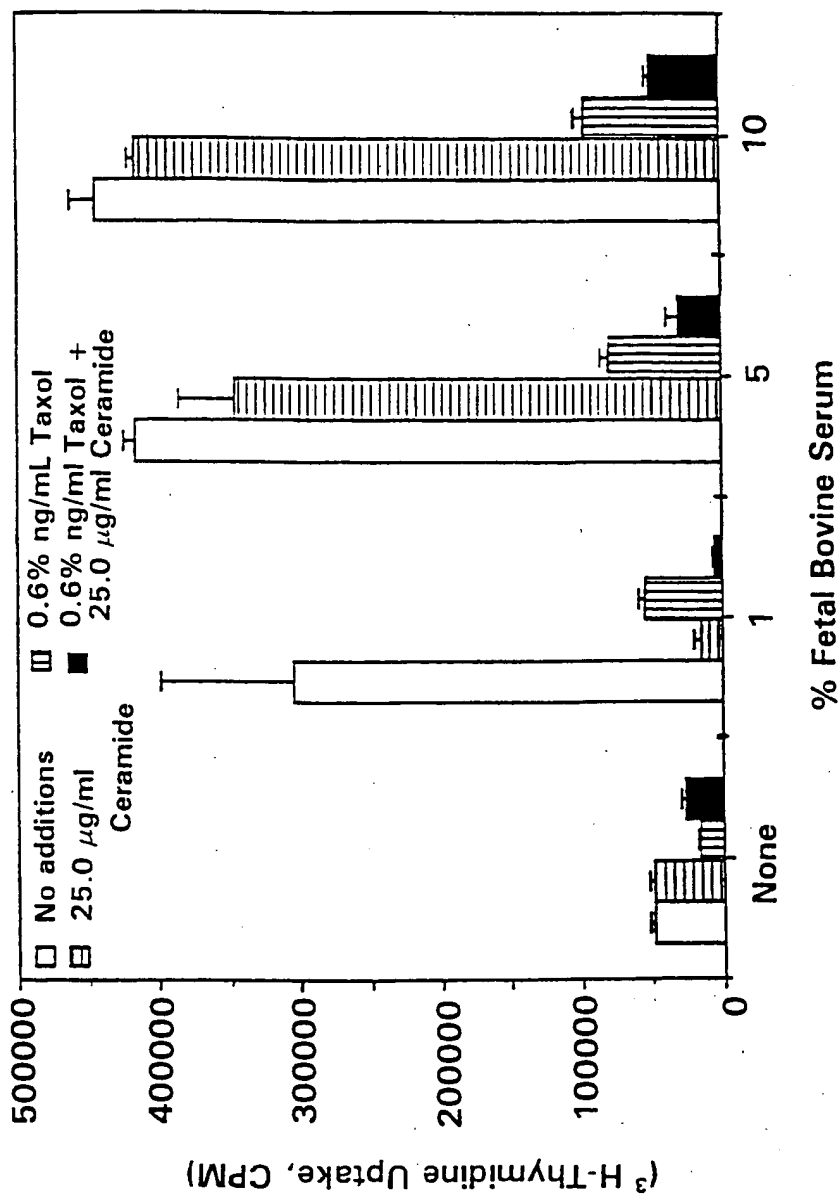


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FIG. 2



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FIG. 3A

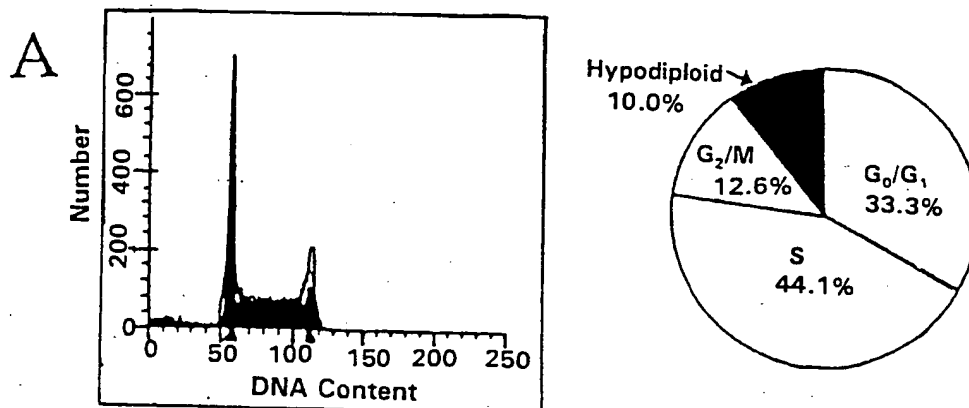
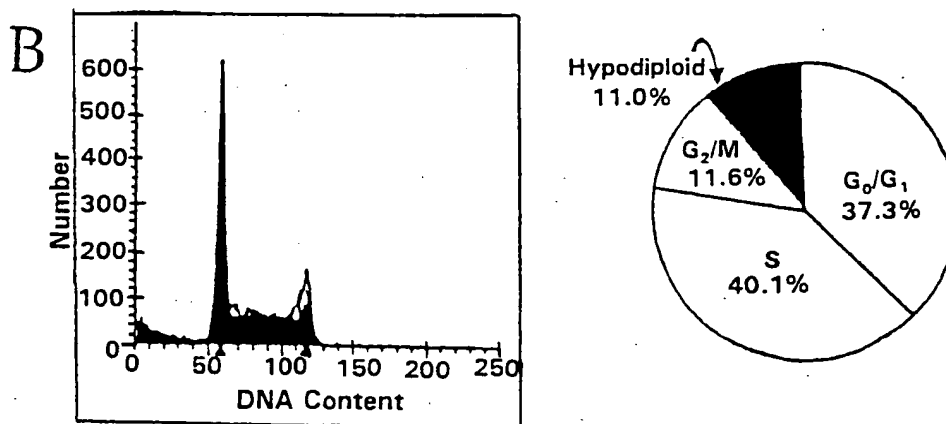


FIG. 3B



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FIG. 3C

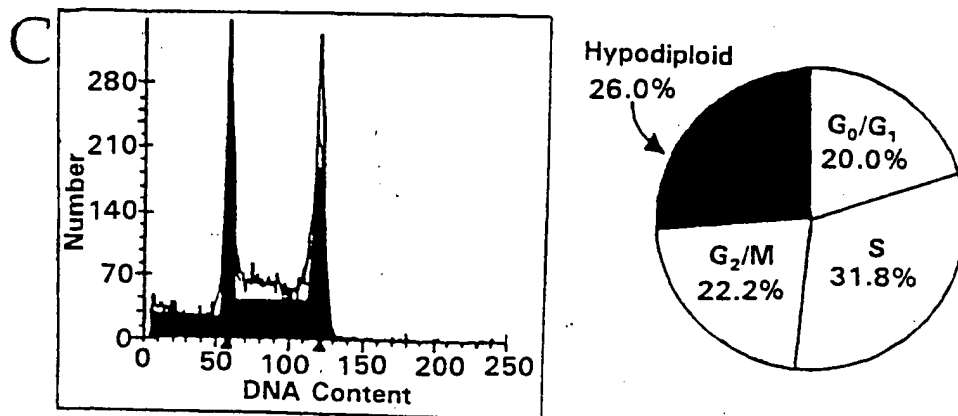
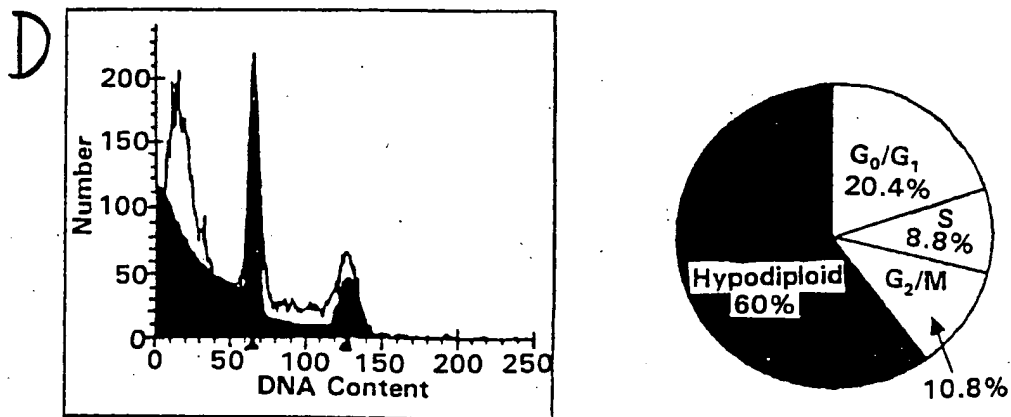


FIG. 3D

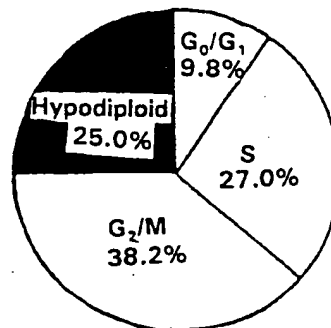
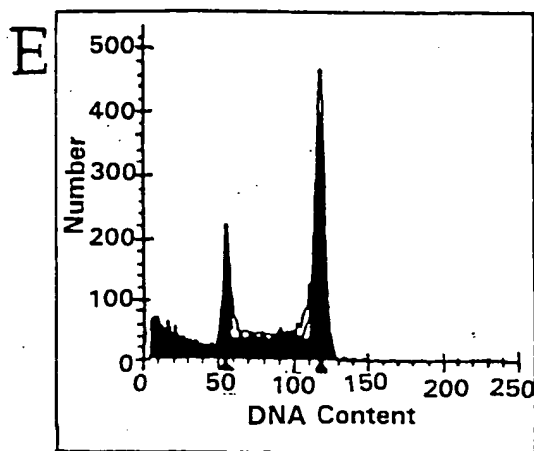


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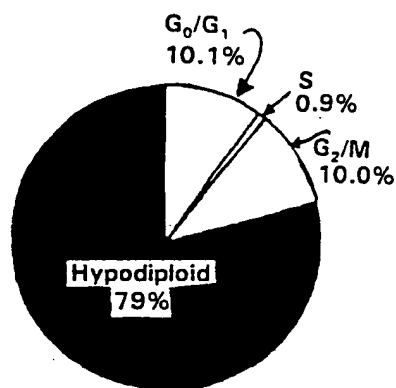
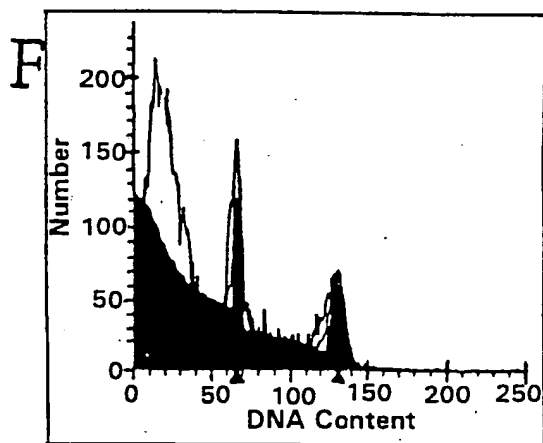
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**FIG. 3E**



**FIG. 3F**



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FIG. 4A

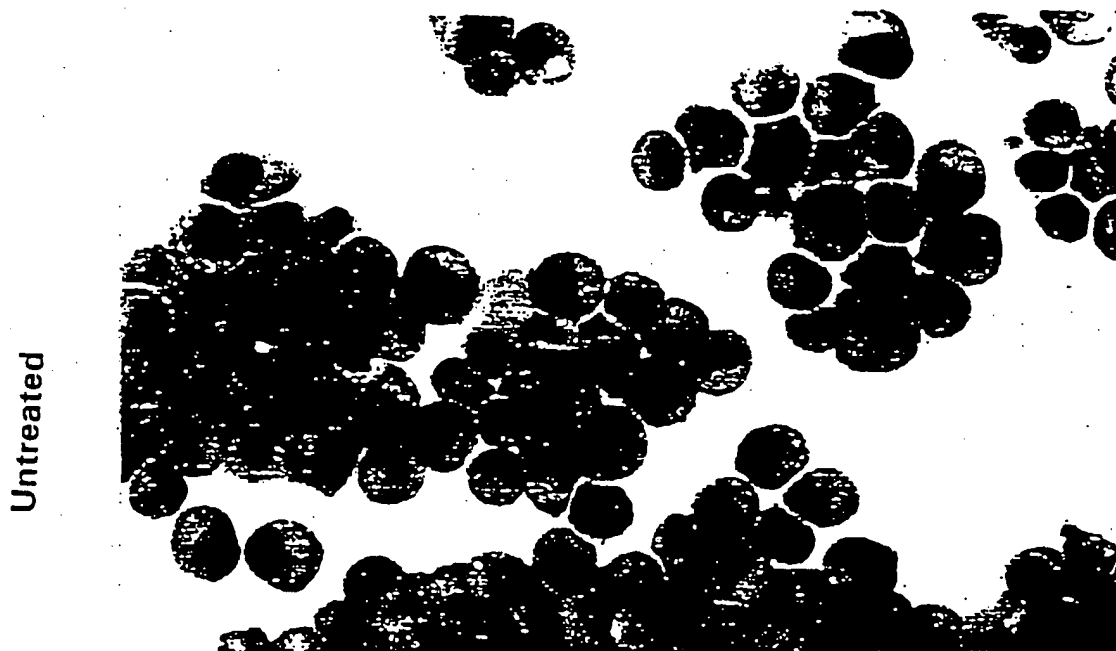
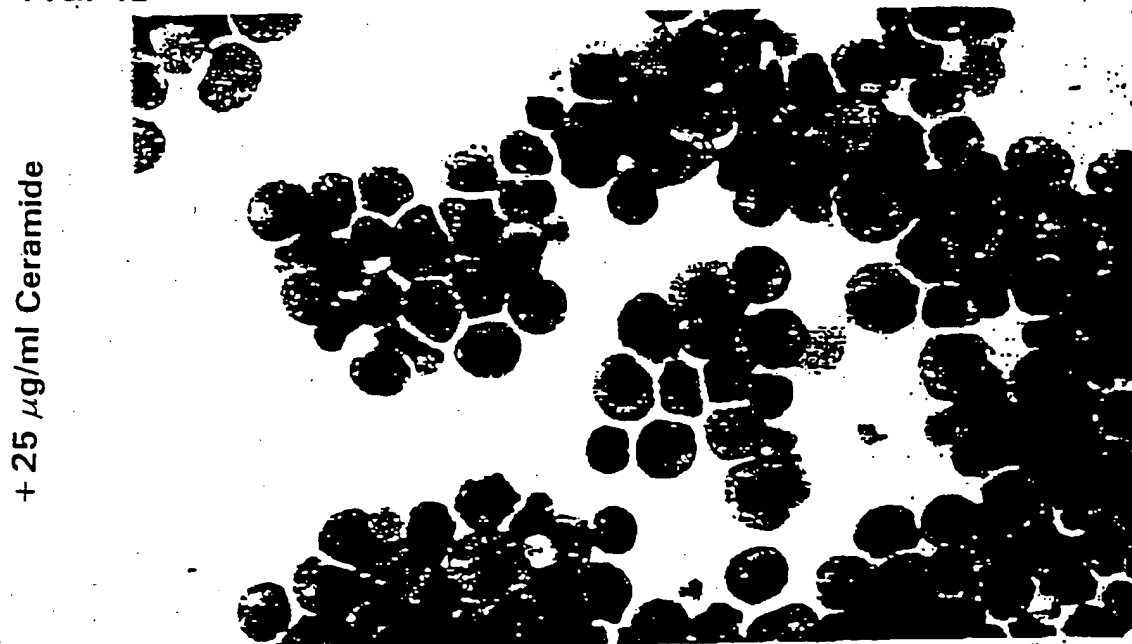


FIG. 4B



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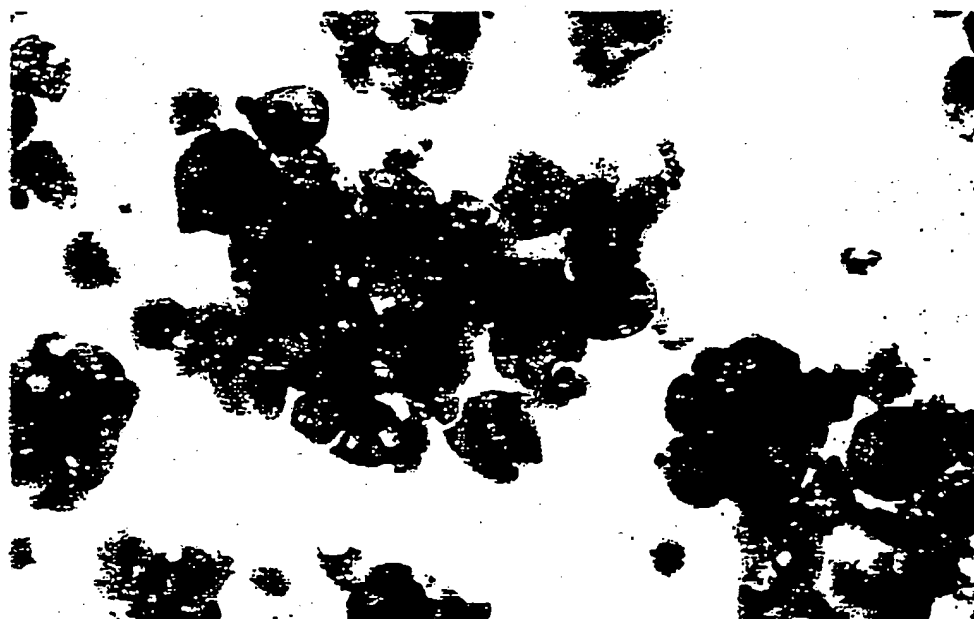
FIG. 4C

+ 0.6 ng/ml Paclitaxel



FIG. 4D

+ 6.0 ng/ml Paclitaxel



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FIG. 4E

0.6 ng/ml Paclitaxel  
+  
25  $\mu$ g/ml Ceramide



FIG. 4F

6.0 ng/ml Paclitaxel  
+  
25  $\mu$ g/ml Ceramide

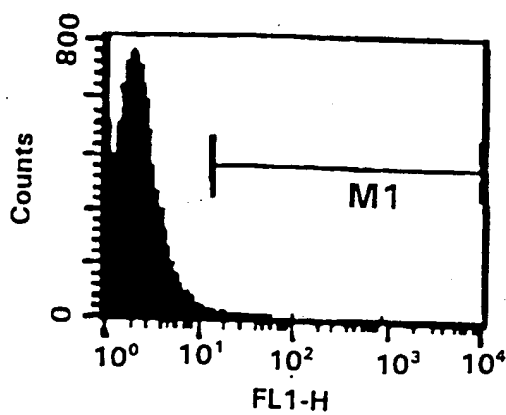


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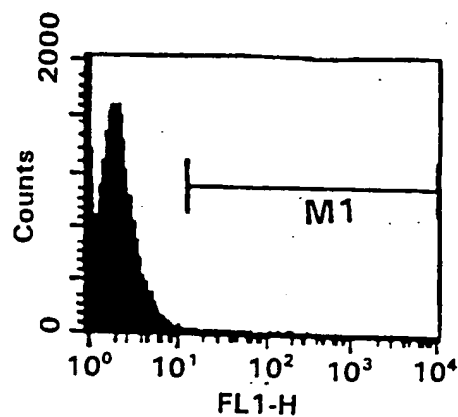
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**FIG. 5A** (24 Hours)



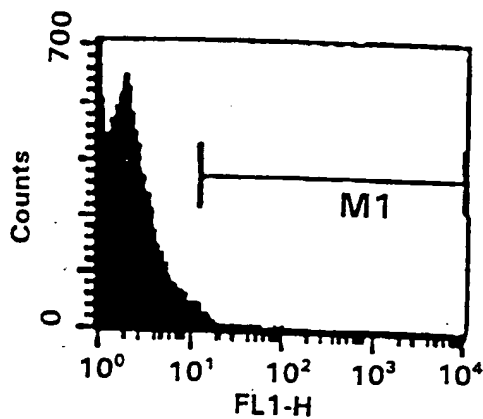
0.93%

**FIG. 5B**



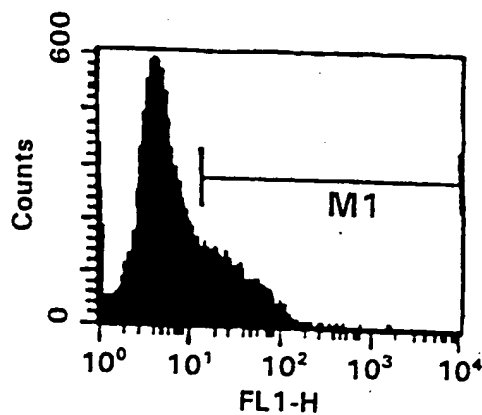
0.64%

**FIG. 5C**



1.16%

**FIG. 5D**



25.49%

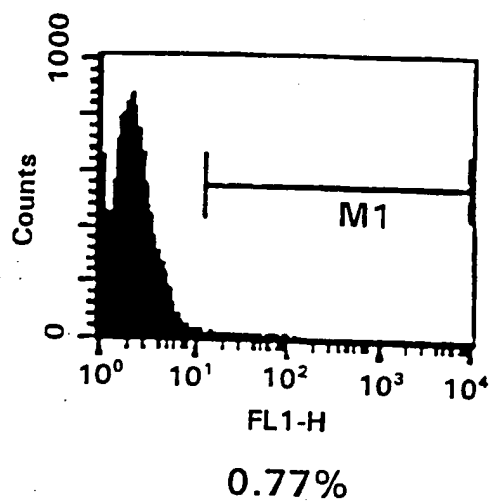


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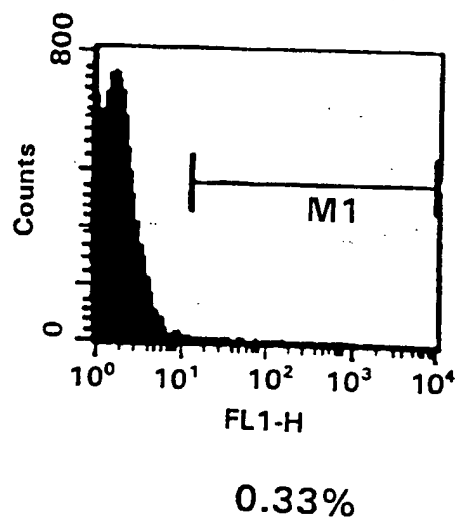
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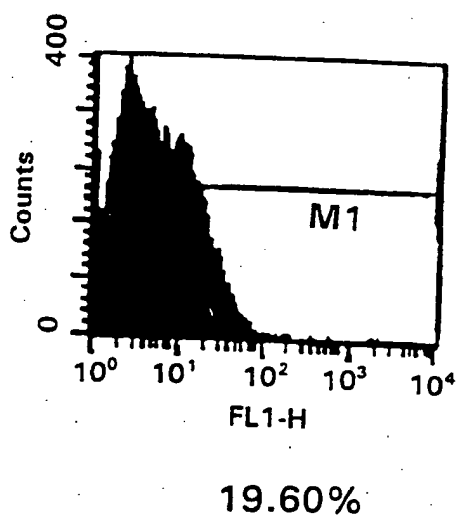
**FIG. 5E** (48 Hours)



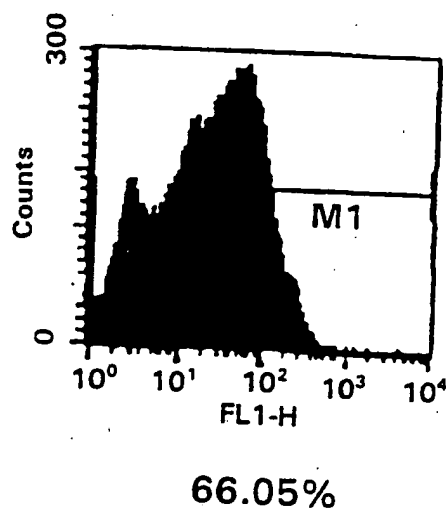
**FIG. 5F**



**FIG. 5G**



**FIG. 5H**

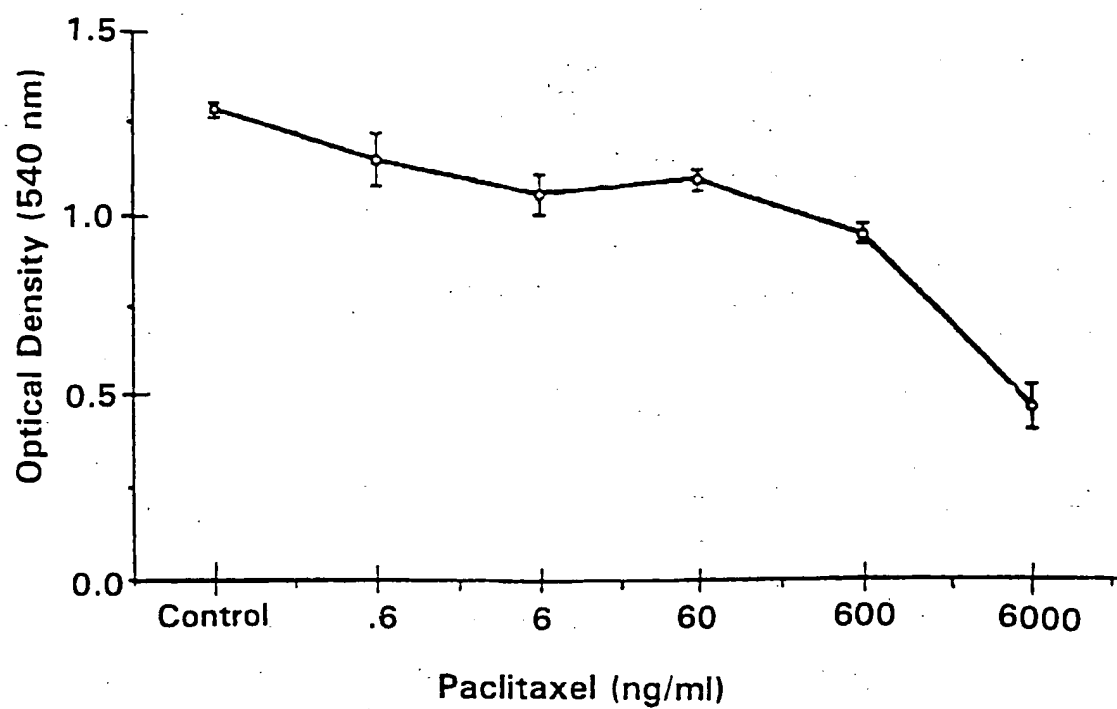


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FIG. 6A

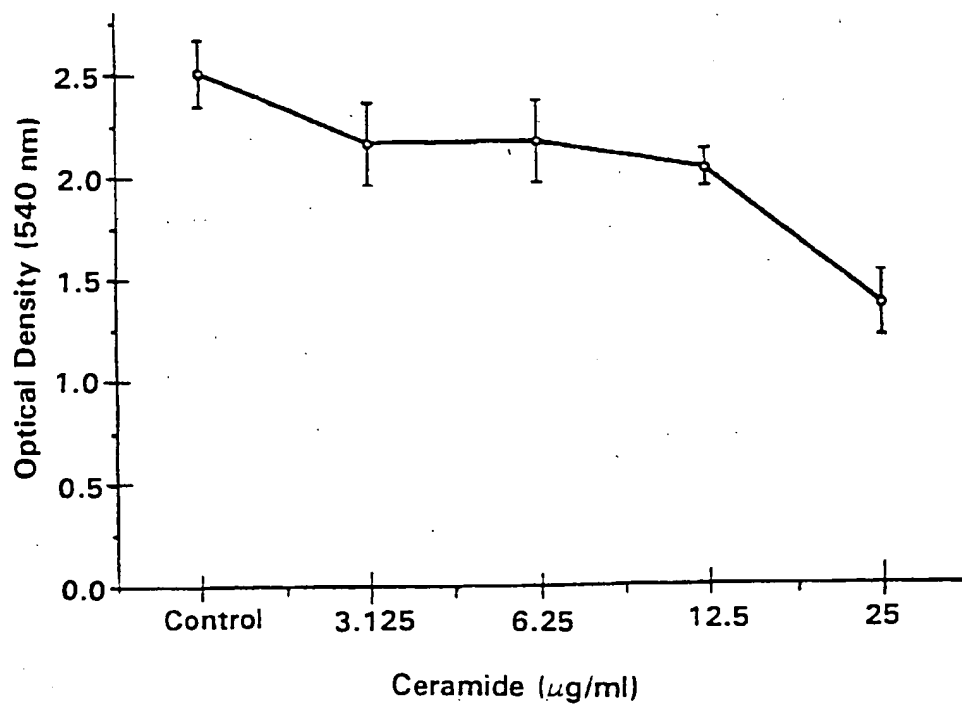


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FIG. 6B

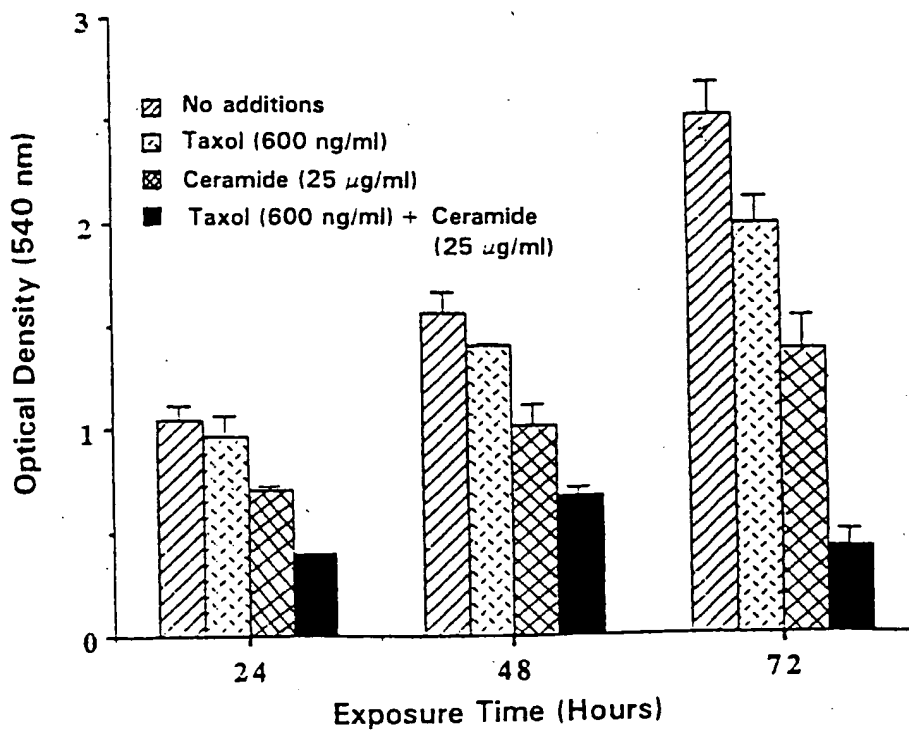


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FIG. 7

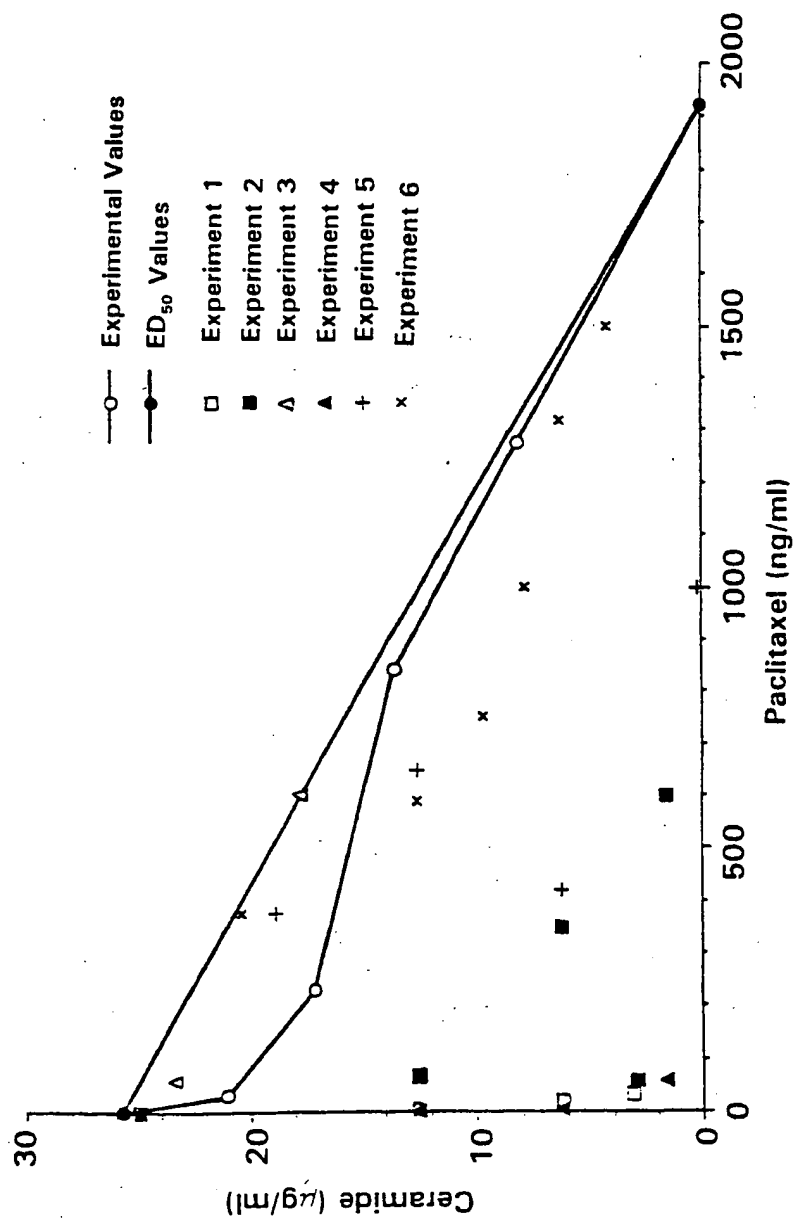


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FIG. 8

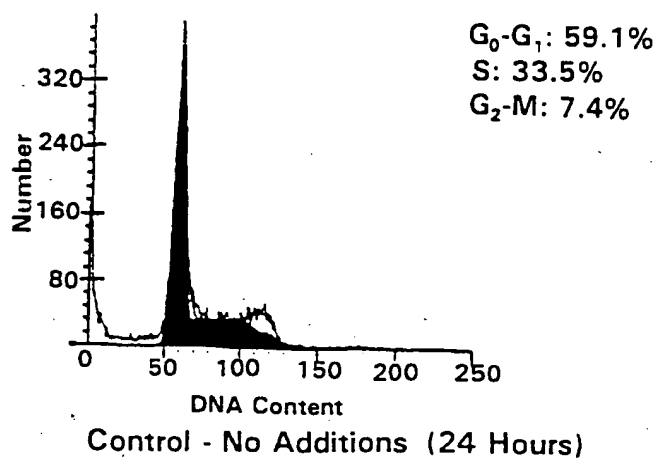


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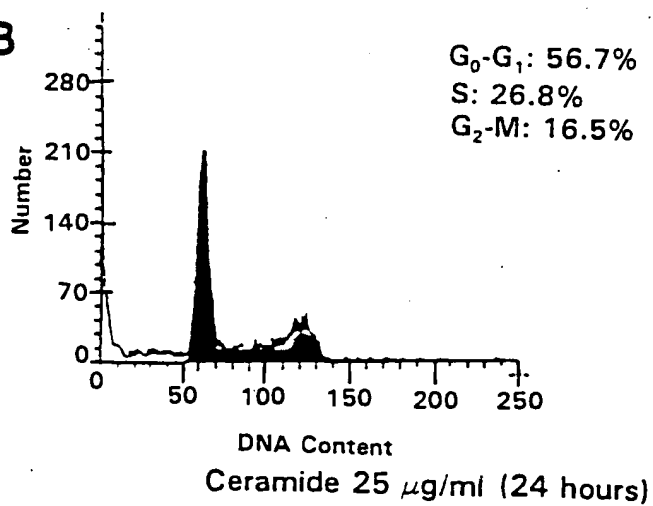
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**FIG. 9A**



**FIG. 9B**

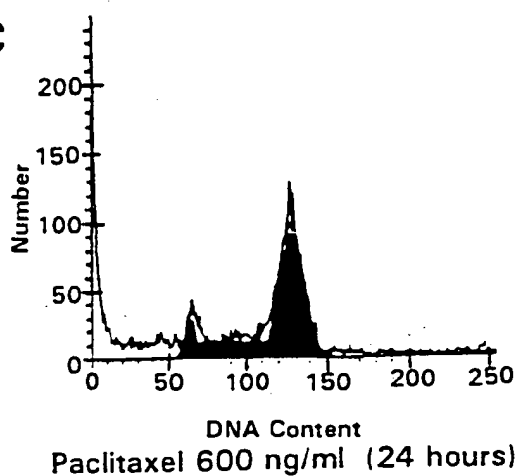


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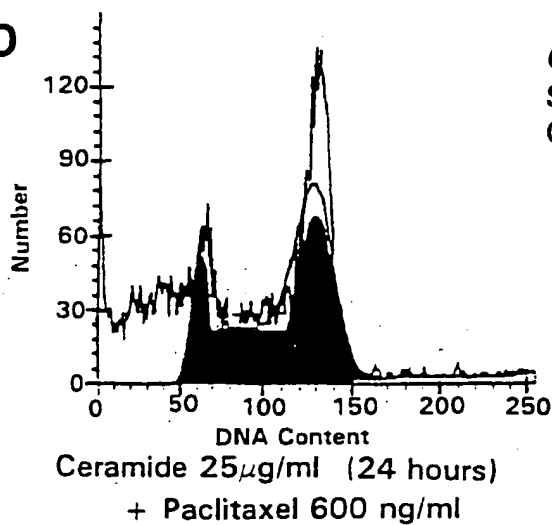
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**FIG. 9C**



G<sub>0</sub>-G<sub>1</sub>: 9.5%  
S: 28.7%  
G<sub>2</sub>-M: 61.8%

**FIG. 9D**



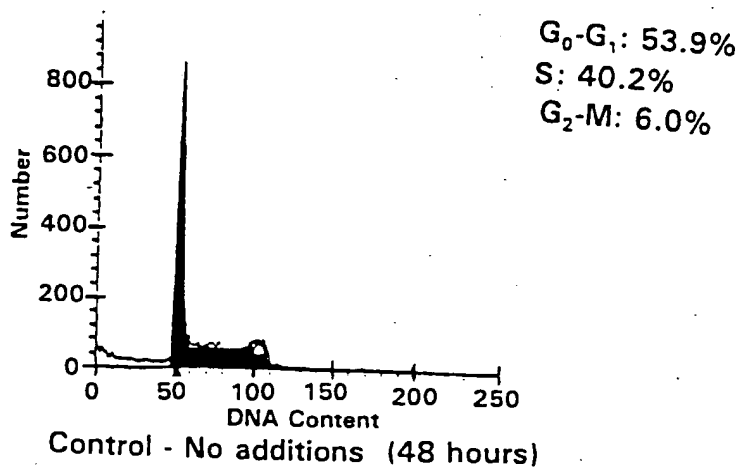
G<sub>0</sub>-G<sub>1</sub>: 17.2%  
S: 37.2%  
G<sub>2</sub>-M: 45.6%

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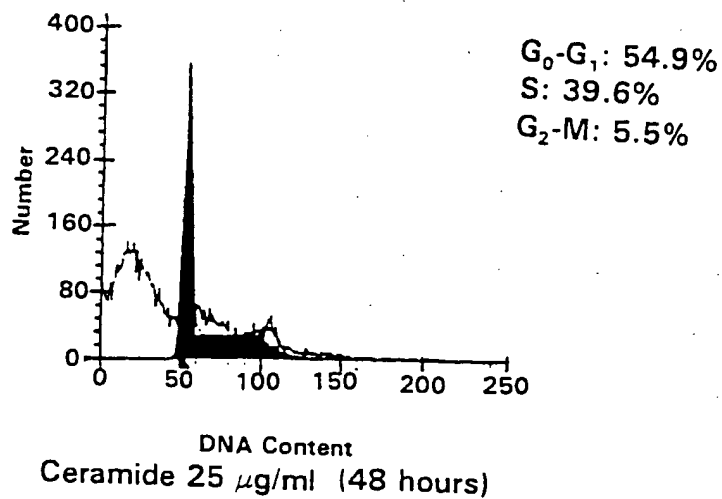
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**FIG. 9E**



**FIG. 9F**



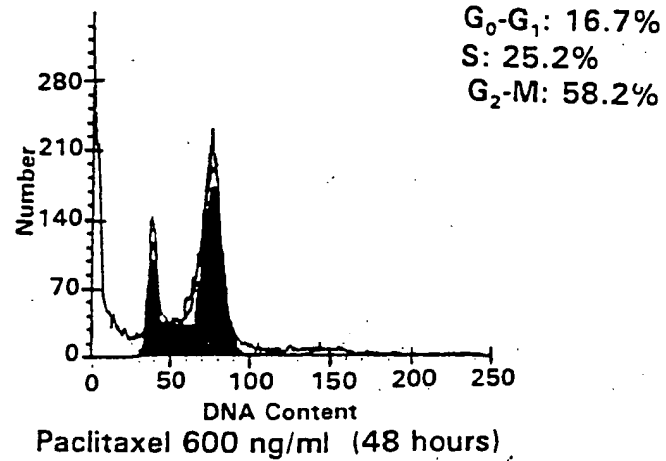


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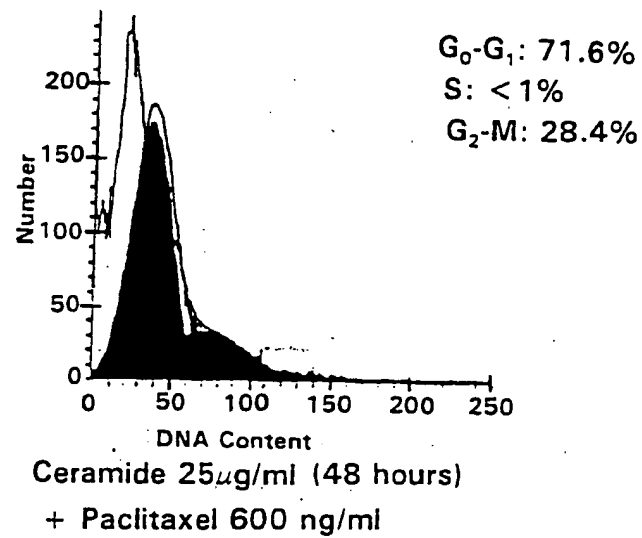
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**FIG. 9G**



**FIG. 9H**



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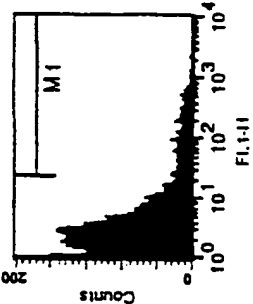
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Time Kinetics Of The Induction Of Apoptosis By Paclitaxel And Ceramide Acting In Combination.

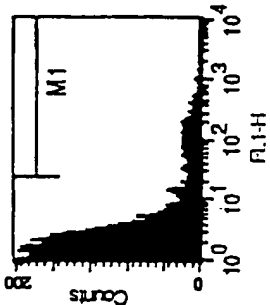
24 Hours Of Taxol And/Or Ceramide Exposure.

FIG. 10A



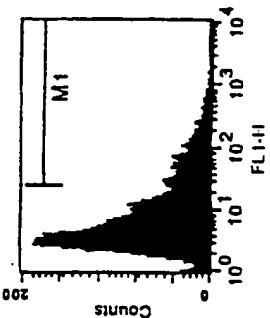
Control - no additions  
Apoptosis: 8.7%

FIG. 10B



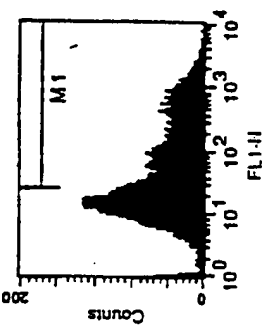
Ceramide (25 µg/ml)  
Apoptosis: 9.7%

FIG. 10C



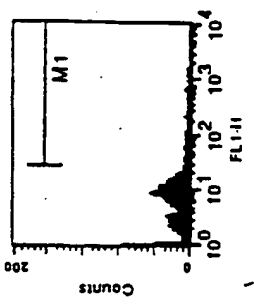
Paclitaxel (600 ng/ml)  
Apoptosis: 18.35%

FIG. 10D



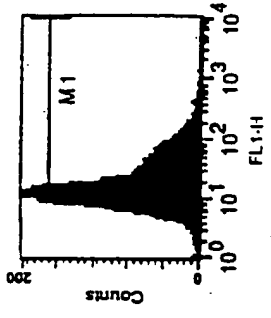
Ceramide 25 µg/ml  
+ Paclitaxel 600 ng/ml  
Apoptosis: 53.7%

FIG. 10E



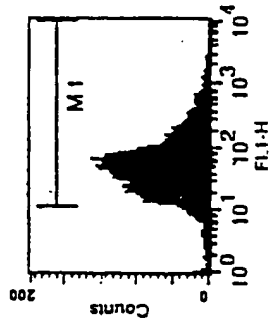
Control - no additions  
Apoptosis: 7.8%

FIG. 10G



Paclitaxel (600 ng/ml)  
Apoptosis: 54.7%

FIG. 10H

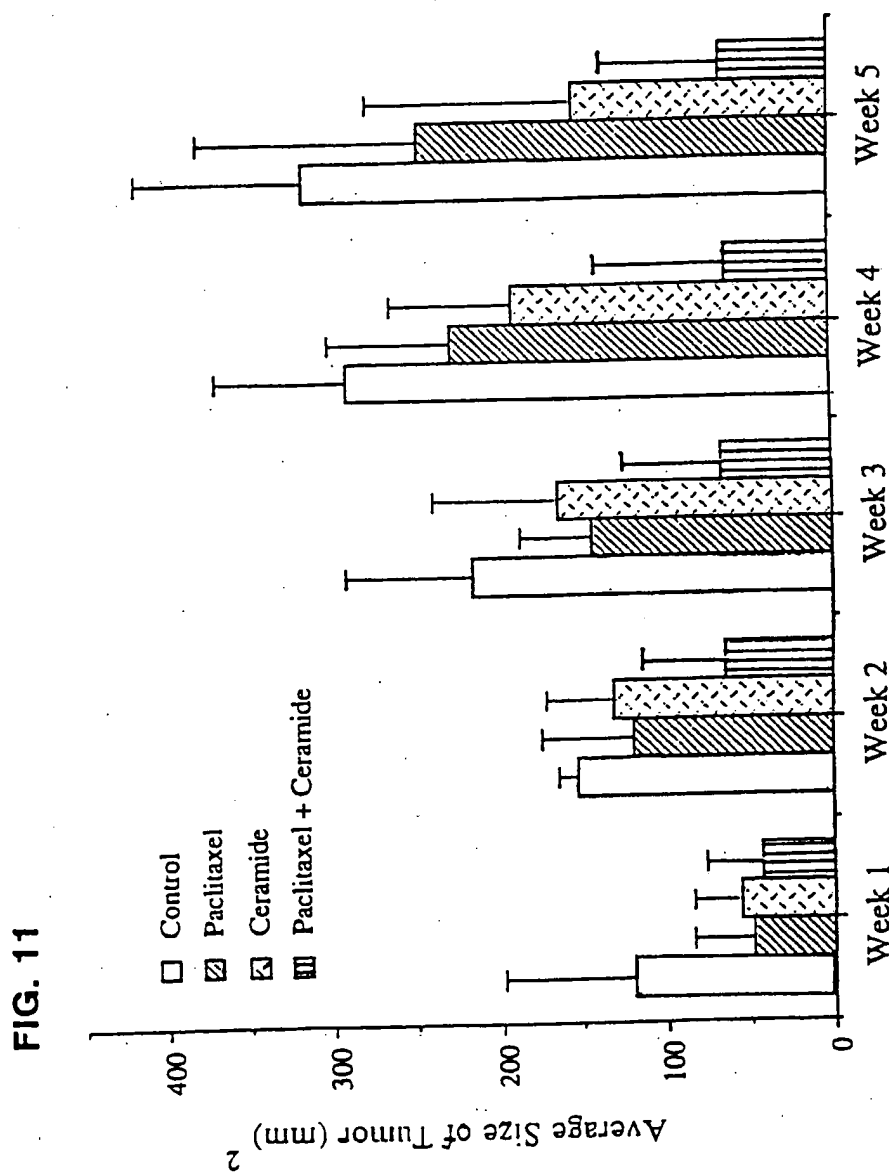


Ceramide (25 µg/ml)  
+ Paclitaxel 600 ng/ml  
Apoptosis: 84.9%

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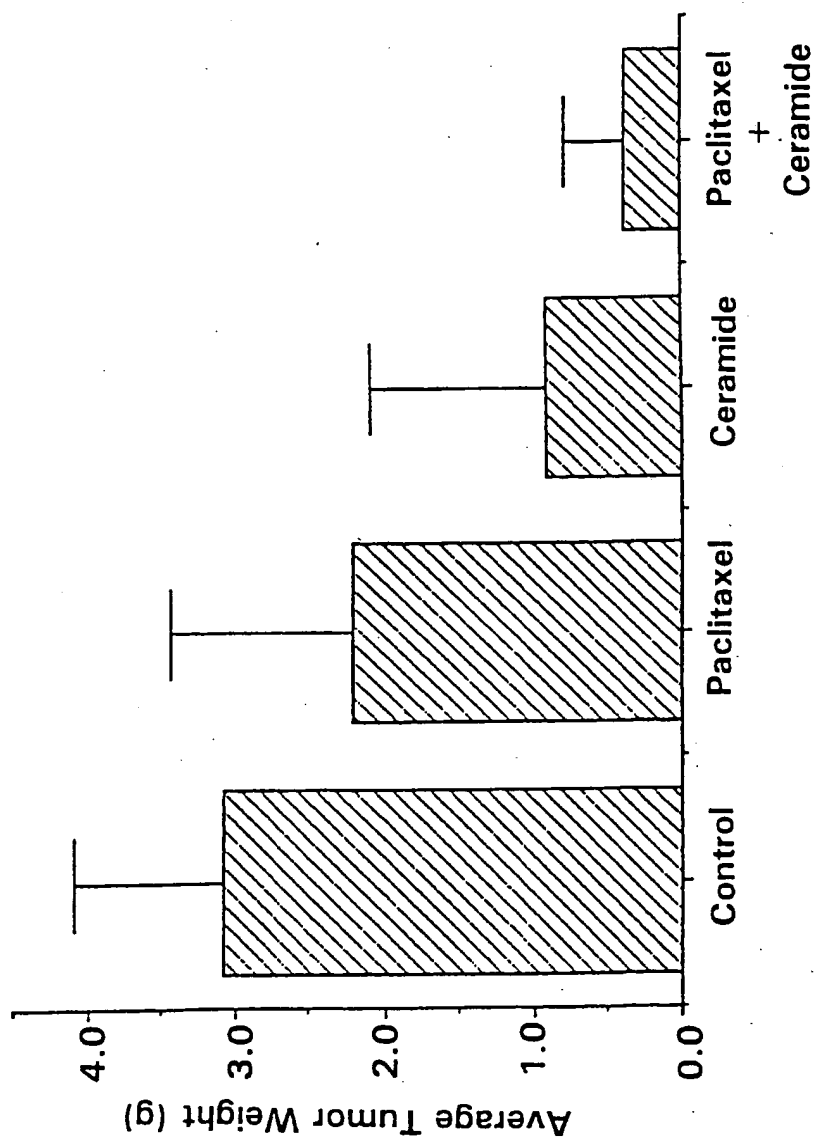


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FIG. 12

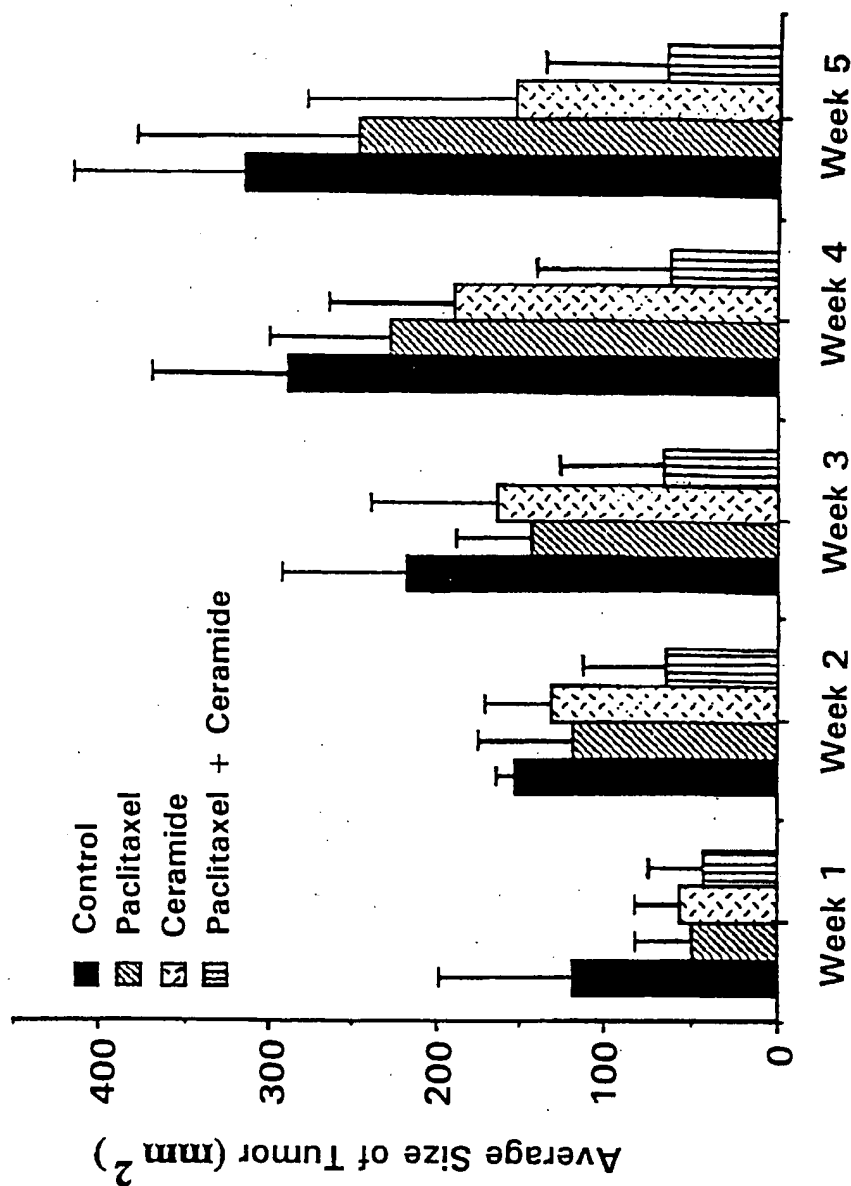


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FIG. 13



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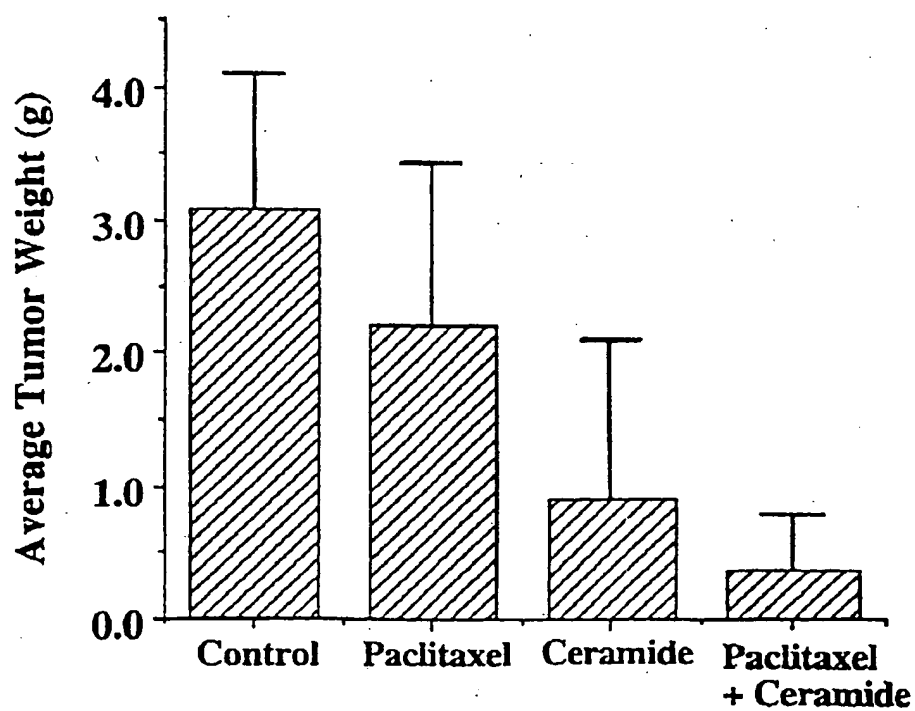


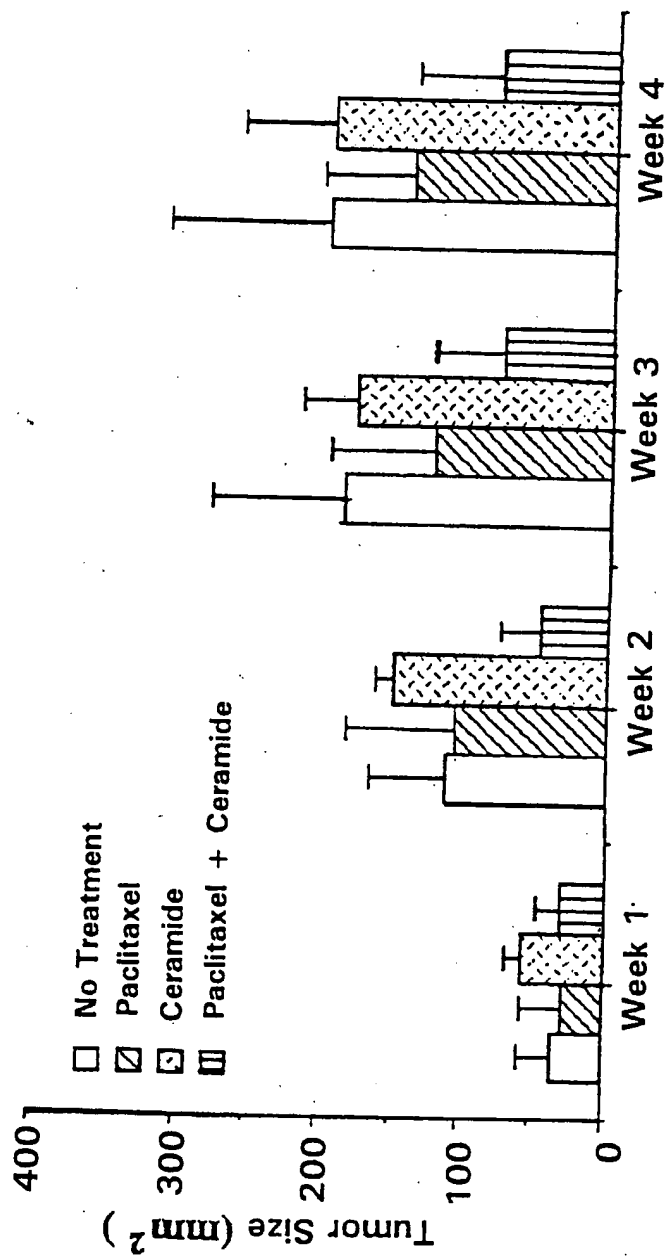
FIG.14

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FIG. 15



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/09440

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC(7) : A61K 33/24; 31/70, 31/335, 31/13, 31/135 US CL : 424/649; 514/34, 449, 645, 648 According to International Patent Classification (IPC) or to both national classification and IPC																				
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) U.S. : 424/649; 514/34, 449, 645, 648 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)																				
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>																				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.																		
Y, P	Database Caplus, An 1999:400782, MYRICK et al., Paclitaxel-induced Apoptosis in Jurkat, A Leukemic T Cell Line, is Enhanced by Ceramide. Leuk. Res. 1999, Vol. 23, No. 6, pages 569-578, Abstract Only.	1-15																		
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.																				
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Date of the actual completion of the international search 22 JUNE 2000		Date of mailing of the international search report <b>11 JUL 2000</b>																		
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer <i>Jerome D. Goldbert</i> JEROME D. GOLDBERT Telephone No. (703) 308-1235																		